

ETIOLOGY AND EPIDEMIOLOGY OF COLLETOTRACHUM SPECIES
RESPONSIBLE FOR ANTIMONONOSE FRUIT ROT AND COLLETOTRACHUM
CHROMI ROT IN STRAWBERRY

By

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To my daughter, Maria, For here at the edge of an old century and the beginning of a new one, they let our hearts be light.

To Maria, her mother, who finds God in also my parents in the spirit for a life full of sharing, social responsiveness, and love.

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TABLE OF CONTENTS

	page
ACKNOWLEDGMENTS	iii
LIST OF TABLES.....	vii
LIST OF FIGURES	viii
ABSTRACT	ix
CHAPTERS	
1 INTRODUCTION	1
2 REVIEW OF LITERATURE.....	4
Sericulture Industry and Production.....	4
Sericulture Anatomy and Morphology	5
Colletes (<i>Colletes</i> spp., <i>Colletes</i> , <i>Taxonomy</i> , and <i>Plant Specificity</i>)	11
<i>Colletes</i> (<i>Colletes</i> spp.) the Sexual Stage	12
<i>Colletes</i> (<i>Colletes</i> spp.) as Sericulture the Polysystem	15
3 OVERWINTER SURVIVAL FOR POTENTIAL ENDOCA OF COLLECTOTRACKDOWN ROT IN BARRED STRAWBERRY CROPS	24
Introduction.....	24
Materials and Methods.....	26
Results	34
Discussion	35
4 ETIOLOGY AND POPULATION GENETICS OF COLLECTOTRACKDOWN SPECIES THAT CAUSE CROWN ROT AND ROOT ROT OF STRAWBERRY	45
Introduction.....	45

Materials and Methods.....	48
Isolates.....	48
Myxomas Production and DNA Extraction.....	50
ITS Primers.....	50
Pathogenicity Tests.....	50
RAPD and Microsatellite Markers.....	54
Data Analysis.....	55
Results.....	56
Species-Specific Primers.....	56
Pathogenicity Tests.....	57
RAPD or Microsatellite Markers.....	58
Discussion.....	64
7. CONCLUSION.....	72
APPENDIX.....	
RAPD AND MICROSATELLITE MARKERS.....	76
REPETO-PCR.....	89
GEOGRAPHICAL SKETCH.....	95

LIST OF TABLES

Table	Page
Table 1 Isolates of <i>Colletes chrysanthemi</i> spp. collected from detrital strawberry leaves (June 1-2000 in both east and west-central Florida)	10
Table 2 Isolates of <i>Colletes chrysanthemi</i> spp. obtained from non-strawberry hosts and used for comparison with <i>Colletes chrysanthemi</i> spp. recovered from symptomatic strawberry leaves	20
Table 3 Adh1, frequencies of 34 putative molecular loci generated with RAPD and corresponding markers from three subpopulations of <i>Colletes chrysanthemi</i> grown on 1C glaucoparasitica obtained from a commercial strawberry farm in west-central Florida	42
Table 4 Percentages of total loci for 33 putative molecular loci generated with RAPD and corresponding markers from three subpopulations of <i>Colletes chrysanthemi</i> grown on 1C glaucoparasitica (Dover P-1, Dover P-2, and Dover P-3 1C glaucoparasitica) and one set of isolates from one (Dover P-3 1C) commercial strawberry farm in west-central Florida	44
Table 5 Presence (+) / absence (-) of putative molecular loci ordered by molecular size (not generated by PCR with (GAGCA) ₁₂ and (ACTG) ₁₂ , primers from crown rot isolates of 1C glaucoparasitica as identified by ITS primers)	56
Table 6 Presence (+) / absence (-) of putative molecular loci ordered by molecular size (lig) generated by PCR with (GAGCA) ₁₂ and (ACTG) ₁₂ , primers from crown rot isolates of 1C glaucoparasitica as identified by ITS primers	72
Table 7 Presence (+) / absence (-) of putative molecular loci ordered by molecular size (lig) generated by PCR with (GAGCA) ₁₂ and (ACTG) ₁₂ , primers from crown rot isolates of 1C glaucoparasitica as identified by ITS primers	78
Table 8 Presence (+) / absence (-) of putative molecular loci ordered by molecular size (lig) generated by PCR with (GAGCA) ₁₂ and (ACTG) ₁₂ , primers from crown rot isolates of 1C glaucoparasitica or 1C anemum (*) as identified by ITS primers	79

Table 9	Presence (+)absence (-) of positive molecular loci ordered by molecular size (bp) generated by PCR with OPC-4 and (TCC) ₁ primers from seven rat isolates of <i>C. glaberrimus</i> as identified by ITS primers	80
Table 10	Presence (+)absence (-) of positive molecular loci ordered by molecular size (bp) generated by PCR with OPC-4 and (TCC) ₁ primers from seven rat isolates of <i>C. glaberrimus</i> as identified by ITS primers	81
Table 11	Presence (+)absence (-) of positive molecular loci ordered by molecular size (bp) generated by PCR with OPC-4 and (TCC) ₁ primers from seven rat isolates of <i>C. glaberrimus</i> as identified by ITS primers	82
Table 12	Presence (+)absence (-) of positive molecular loci ordered by molecular size (bp) generated by PCR with OPC-4 and (TCC) ₁ primers from seven rat isolates of <i>C. glaberrimus</i> or <i>C. aestivum</i> (*) as identified by ITS primers	83
Table 13	Presence (+)absence (-) of positive molecular loci ordered by molecular size (bp) generated by PCR with (GACCA) ₁ and (ACCTG) ₁ primers from three rat isolates of <i>C. aestivum</i> as identified by ITS primers	84
Table 14	Presence (+)absence (-) of positive molecular loci ordered by molecular size (bp) generated by PCR with (GACCA) ₁ and (ACCTG) ₁ primers from three rat isolates of <i>C. aestivum</i> as identified by ITS primers	85
Table 15	Presence (+)absence (-) of positive molecular loci ordered by molecular size (bp) generated by PCR with OPC-4 and (TCC) ₁ primers from three rat isolates of <i>C. aestivum</i> as identified by ITS primers	86
Table 16	Presence (+)absence (-) of positive molecular loci ordered by molecular size (bp) generated by PCR with OPC-4 and (TCC) ₁ primers from three rat isolates of <i>C. aestivum</i> as identified by ITS primers	86
Table 17	Presence (+)absence (-) of positive molecular loci ordered by molecular size (bp) generated by PCR with (GACCA) ₁ and (ACCTG) ₁ primers from isolates of <i>C. jaguensis</i> from laboratory or other California loci spp. isolated from non-laboratory hosts as identified by ITS primers	87
Table 18	Presence (+)absence (-) of positive molecular loci ordered by molecular size (bp) generated by PCR with OPC-4 and (TCC) ₁ primers from isolates of <i>C. jaguensis</i> from laboratory or other California loci spp. isolated from non-laboratory hosts as identified by ITS primers	88

LIST OF FIGURES

Figure	Page
Figure 1 Frequency of recovery of <i>Callosorhiza planyptoides</i> from buried strawberry roots: (A) Recovery from surface sterilized roots segments buried at 13 cm in 1994 and 5 and 13 cm in 1995 (B) Recovery by obtaining plating of ground straw roots buried at 5 and 13 cm in 1995 (C) Percentages of roots at which <i>C. planyptoides</i> was detected using from either sterilized segments or dilution plating of ground roots 14	
Figure 2 Weekly precipitation (A), weekly average air temperature (B), and weekly soil temperature (C) at the Gulf Coast Research and Education Center in Dade County, Florida during the summer and fall of 1993 and 1994. No soil temperature data was recorded between 23 July and 6 August 1994 18	
Figure 3 Average weekly soil water potential recorded at 5 and 13 cm during the summer and fall of 1994 at the Gulf Coast Research and Education Center-Dade 20	
Figure 4 Dendrogram based on RAPD analysis of 12 isolates of <i>Callosorhiza</i> spp. showing relationships between <i>Callosorhiza</i> from straw root isolates (Straw Root) and strawberry fruit rot isolates (Straw Root) of strawberry and 13 non-strawberry hosts (Glenn Root with striped leaves). Two arrows at the cluster of fruit rot isolates correspond to the presence of two isolates of <i>C. anthonii</i> isolated from the crowns of plants that did not produced typical symptoms of <i>Callosorhiza</i> -straw-rot or pathogeny-rot. Dendrogram constructed using the unweighted pair group method with arithmetic average (UPGMA) based on Dice similarity coefficient 29	

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Colletotrichum species cause serious diseases in strawberry worldwide and little is known about their ethology and epidemiology. The purpose of this study was to evaluate the potential for *Colletotrichum* spp. to overwinter in plant debris under field conditions in Florida and to investigate the population structure of these fungi and etiology of the diseases they cause. Strawberry crowns naturally infected with *Colletotrichum* spp. were placed inside cloth bags, buried in the field, and then recovered over a period of 6 months during two consecutive years. After recovery, the crowns were placed onto a *Colletotrichum* root-selective medium and quantified by colony and spore morphology, and by PCR using primers from the ribosomal DNA internal-transcribed spacer (ITS) region. Of 411 isolates of *Colletotrichum* spp. recovered, both were *C. gloeosporioides* and 95. *C. acutatum*. Recovery of *Colletotrichum* spp. began 10-14 days 1 or 4 weeks after burial and no *Colletotrichum* spp. were detected after 90 days of burial.

Because the time between seasons is typically more than 170 days, these data support the hypothesis that the isolation of *C. glaucosperma* from plant debris does not play a major role as *Colletotrichum coccis* rot epidemics in Florida. In a second experiment, monoconidial isolates of *Colletotrichum spp.* were subcultured to confirm the ecology of *Colletotrichum coccis* rot (1 H isolates) and anastomosis from rot (20 isolates), and assess the genetic diversity of the pathogen populations involved in these diseases. Using randomly amplified polymorphic DNA (RAPD) analysis, DNA fragments between 0.50 to 1.0 kb in size were converted into a binary character matrix and analyzed using congruent algorithms. Few polymorphisms were observed among that rot isolates. In contrast, seven rot isolates were highly polymorphic. ITS primers identified all that rot isolates as *C. coccis*. Among seven rot isolates, 85% of tested isolates were identified as *C. glaucosperma* and only 15% as *C. coccis*. The RAPD and ITS data analyses suggested that anastomosis that rot is caused by an internal (clonal) population of *C. coccis* whereas *Colletotrichum coccis* rot is caused by a highly polymorphic sexually reproducing population of *C. glaucosperma*.

CHAPTER I INTRODUCTION

The ability to cause wilts in a wide range of plants (including important monocotyledonous and dicotyledonous crops) has placed *Colletotrichum* spp. among the most important pathogen groups especially in sub-tropical and tropical regions (Baker and Jeger 1992). *Thioperidiosis*, produce symptoms, commonly known as wilts, occurs in an equally diverse group of types of host plants including fruits, leaves, and stems (Baker and Jeger 1992). Typical anthracnose symptoms are generally described as localized, slightly raised lesions often having a sunken-pink sticky spot centers (Baker and Jeger 1992; Howland et al. 1992).

Within the United States, strawberry (*Fragaria x ananassa* Duchesne) is considered a major crop (Howard et al. 1992). However, among Florida vegetable strawberry, multi-crop production value with a total farm gate revenue value greater than US\$400 million for the 1992 to 1998 season (Florida Department of Agriculture and Consumer Services, 1999).

In strawberry, *Colletotrichum* spp. cause important diseases that affect roots, leaf, pedicels, stolons, fruit, and petioles (Howard et al. 1992; Howard and Allington 1993; Wilson, 1995) and can cause significant yield losses in most strawberry production areas worldwide (Howard and Allington, 1993; Olson-Rodell and Wilson, 1995; Wilson et al. 1992). In Florida, *Colletotrichum* spp. have caused up to 80% crop loss in nursery and growing fields (Howard et al., 1992; Howard and Allington 1993). *Colletotrichum*

crustacean *C. glaucoperculatus* and *C. fragilis* are the most frequently identified pathogens associated with ulcers on starfishes (Freeman and Katers, 1997; Howard et al., 1992) and it has been suggested that a complex of these pathogens is responsible for the various diseases observed in production fields (Howard et al., 1992).

Although it is generally accepted that splash or wind-driven rain dispersed animals accounts for most of the water-borne dispersal of *Colletotrichum* spp., it is likely that some results of *Colletotrichum* spp. are dispersed through the field by harvesting and other farm operations (Gibson and Martin, 1991; Leggett, 2000; Wilson, 1999). Although the role played by insects as dispersal or overwintering hosts is clear, other epidemiological questions about diseases caused by *Colletotrichum* spp. and the taxonomy of the pathogens (both anamorphic and teleomorphic stages) remain to be resolved (Bailly and Leggett, 1992). Because of the difficulty in separating *Colletotrichum* spp., determining the etiology of these diseases has been difficult. Epidemiology information is critical for the survival of the fungus as well as the source of initial infection for underwater pathogens (Howard et al., 1992; Leggett, 1992; Smith and Black, 1999). To improve the management of these diseases, elucidating the etiology of these diseases is critical. If distinct species are responsible for different diseases on starfishes, then biological differences between the species could be exploited to improve their control.

The goals of the research for this dissertation were to improve the understanding of the etiology, epidemiology, and biology of *Colletotrichum* spp. that cause crown rot and flat rot on starfishes in Florida. This research focused on two major water-borne pathogens for *Colletotrichum* spp. to understand its plant diseases and serve as a source of

primary reservoir for avian red epidemics in Florida, and the ecology and population genetics of *Colasporidium* spp. responsible for bird red and avian red epidemics in Florida.

CHAPTER 2 REVIEW OF LITERATURE

Strawberry Industry and Production

Strawberry (*Fragaria x ananassa* Duchesne) is among the most profitable of fresh fruit, making the production and commercialization of good quality fruit difficult. Despite this, worldwide production has been increasing especially in the northern hemisphere (Hammami and Lopez, 2000; Marshall, 1982). The United States is the largest producer with 30% of the world's crop followed by several European and Asian countries including Japan, Italy, Japan, and the Korean Republic (Hammami, 1999; Hammami and Lopez, 2000). Strawberry production in the United States has steadily increased since the 1970's even though the total under production has remained relatively unchanged during the last 35 years with ca. 21 000 ha (Hammami, 1999). The rise in production is due mostly to crop increases in marketable yield in California and Florida, which along with Oregon and North Carolina are the most important production areas in the USA (Hammami, 1999). In Florida, the farm-gate revenue for the strawberry-crop during the 1997 to 1998 season increased 80% over the 1990 to 1991 season (\$361.2 million, vs. \$201.3 million, Florida Department of Agriculture and Consumer Services, 1998).

The primary market for Florida fresh strawberries extends across the U.S. and into southern Canada (Florida Department of Agriculture and Consumer Services, 1998; Maynard et al., 2000). A majority of the production in Florida is located in west-central

Florida, around the Gainesville City area. The climatological advantage of the region lies in its mild winter temperatures. In west central Florida the average low temperature for December through February is 18-20°C. Strawberry plants are induced to dormancy by temperatures below 7.2° C and require temperatures above 15° C for flower bud formation. At higher temperatures and depending on cultivar, the annual photoperiod for floral induction is 8 to 12 h (Hawesick, 1999). Strawn savings occurs at temperatures below 7.2° C and, fruit production is maximized if plants are entering vernal (Hickelshy and Rose, 1992). Additionally, it has been demonstrated the rate of floral development is strongly related to temperature, as is the rate of fruit production (Hawesick, 1999). Therefore, winter temperatures found at west central Florida are the most suitable temperatures for winter production of strawberries in the U.S.

Conventionally strawberry plants are closely produced (Hawesick, 1999) and in Florida, grass-top-strawplants that mature as in the northeastern US or southeastern Canada are not available from late September through early May either. Seedlings are transplanted in the April through August, plants establish beds and overhead irrigation for 10 to 14 days during establishment (Leyser, 2002; Maynard et al., 2002). Plant are typically harvested from late November through early April (Florida Department of Agriculture and Consumer Services, 1999; Maynard et al., 2002) although some fruit are harvested as late October and late April.

At the end of the season, growers destroy the plants by applying paraquat or mowing the tops off the plants. Some growers will plant spring vegetable crops into the existing beds after killing the strawberry plants. From April to late May, a grass or legume-soybean crop is typically planted. During August or early September, the cover

crop is transported and most beds are formed and integrated with a network of methylotrophic fungi and rhizomorphs. In Florida, only the beds are integrated and mycelium can grow in the same location every season without crop rotation (Lagout, 2008).

Species within genus *Pezizaria* have distinct sexual/sexual dimorphism in growth rate at some of the species, and reproduce both sexually and asexually (Hawcock, 1990). Genetic exchange among the species is relatively unrestricted (Gelfelt and Mass, 1990; Hawcock, 1990). Cultural strawberry resulted from a cross between *P. albidissima* (100-10 clonemorphous) and *P. repens* (100) (Hawcock, 1990; Maynard et al., 2002). The most broadly distributed cultural species is the diploid *P. repens* found in North and South America, Europe, Asia, and Brazil and is considered to be the progenitor of modern cultivated strawberries (Hawcock, 1990).

Most strawberry breeding programs have been focused on improve fruit quality (such as incorporation of flavor and aroma), resistance to biotic and abiotic stress, and the ability to mature and use structures that under a variety of climatic regions (Dewdney, 1999; Gelfelt and Mass, 1990; Lawrence et al., 1998). Less time and resources have been dedicated to breed or screen new strawberry cultivars for disease resistance and only a few diseases have caused significant damage from berries (Dewdney-Rothman et al., 1999; Hawcock, 1990; Mass, 1990). Resistance to *Colletotrichum* spp., *Diplonecyrtis caribaea*, *Sclerotinia* spp., *Phytophthora* spp., *Ascochyta* spp. and *Puccinia* spp. have been described among strawberry cultivars under greenhouse and field conditions (Dewdney, 1999; Dewdney-Rothman et al., 1999; Gelfelt et al., 1991; Mass, 1990). Resistance of strawberry plants to *Colletotrichum* spp. is affected by environmental conditions (Dewdney-Rothman et al., 1999; Smith and Black, 1997) and

apparently is unequally controlled by major genes (Smith and Black, 1963). There are reports indicating that expression of disease resistance depended on the strains of the pathogens as well as the type of symptoms produced by the pathogens (Dreyer-Rothbarth 1917; Ogden and Smith, 1961; Smith and Black, 1963). Culture Sweet Charlie, for example, is highly resistant to anthracnose from rot but susceptible to Colletotrichum crown rot (Chandler et al., 1991).

Strawberry Anatomy and Morphology

Strawberry is a perennial in the Rosaceae family and it is considered for some the only vegetable crop in this family (Maynard et al., 2000). However, a large number of ornamental plants and important tree fruits such as apple, pear, cherry, and plum are included in this family (Hessrick, 1998; Maynard et al., 2000). Most species in the Rosaceae family have alternate leaves and stipules, and these may be alternate in the petiole (growing with one side adjacent to the petiole). In the subfamily of the strawberry (Fragarioideae) apocarpous perianth occurs, the achene (embryo into a hard seed) without hard indehiscent fruit. As during these one flower develops, the individual achenes become spatially isolated on the surface of the greatly enlarged receptacle, giving the "fruit" its botanized classification as an aggregate accessory fruit. These dried one-seeded fruits are commonly named as the "seeds" of the fruit (Dudney and Maynard, 1993).

The strawberry plant is characterized by a short (approximately 2.5 cm long) central stem known as the crown from which leaves, stipules, roots, stolons, and modified stems or cymae (inflorescences) emerge (Hessrick, 1999). The crown is composed basically of a continuous central strand of predominantly pericyclethems and meristem tissue (peak in meristem) surrounded by a rhizome-cortexium and the epidermis

lignification of vascular elements during lateral crown growth produces a hard and woody texture in the crown. Structural organs such as roots and leaves originate from the older woody portions of the crown (Callaway and Bingham, 1998).

The leaves, arranged in a tight 2:1 spiral, are bilobulate (ovate-lanceolate) and pinnate (Blascock, 1993). Axillary meristems are located between each leaf and the crown and depending on the environment and nutrient level, these may develop into runners (Callaway and Bingham, 1998). A terminal meristem can develop leaves or become an inflorescence in which case the continuing vegetative crown growth displaces the inflorescence at the apical terminal axis off to one side (Callaway and Bingham, 1998). The small flowers have five sepals, a very short hypandrium (an enlargement of the sep-als) of receptacle bearing on its inside stamens, pistils, and sepals) and an apocarpous gynoecium of many free carpels on an elevated receptacle (Blascock, 1993; Callaway and Bingham, 1998).

Cultivation and Diseases, Pests, and Host Specificity

Extensively, unknown diseases of foliage, stem, and fruit have caused serious losses in many crops worldwide (Agnew, 1997). The term *silver scum*, which literally means "dead silver", was first used as a synonym for scum in France in 1857 to describe silver-like symptoms produced by *Sphaeria aspidium* (teleomorph *Clavus aspidium*) in grapes (Farrar) (Lilja and Kitch, 1988), but later was applied to diseases caused by numerous fungi producing similar silver-black scums such as *Sphaeria*, *Blumeria*, *Gnomonia*, and *Glenomyces* (Agnew, 1997). Anamorphs of these genera, which can be found under the former classification of *Ascomycota* (*Dothiorella*), *Dothiorella* fungi in which conidia are formed within a cavity lined by host tissue),

includes *Heterospora*, *Sphaerospora*, and *Collosporidium* (Hawkerworth et al., 1995; Butler, 1995). Syndromes produced by *Glossosporium* have also been reported in the literature as suberiosis (Hawkerworth et al., 1995). However, the name *Glossosporium*, proposed for *Collosporidium*-like fungi that did not produce spores, has been rejected and fungi associated with this genus is now classified as *Marasmius* spp. *Collosporidium* spp. and others (Hawkerworth et al., 1995; van Aarts, 1937 as quoted by Busby and Jeger, 1992). Today the use of the term suberiosis still retains its etiological implications and is usually associated with fungi that form black, sunken, limited lesions in which suberaceous or subepidermal necrosis, with necrosis at macrofungal centers (usually pink or orange to off-white [often appearing as the host in concentric circles]) are present (Lajunen, 1997; Baranick, 1991; using Stenroos, 1958).

Among fungi associated with suberiosis-type syndromes, the genus *Collosporidium* is considered one of the most important because of its worldwide distribution, and ability to cause both preharvest and postharvest suberiosis, and the magnitude of economic losses in important crops worldwide (Busby and Jeger, 1992; Busby et al., 2000). In 1937, Cooke (cited by Duke, 1953) proposed the genus *Collosporidium* with a single species, *C. boudieri*. Later necrotic, localized, sunken, necrotic, hyaline spores, and dark, angular, subulate setae characterized the fungus. The name *Collosporidium* was accepted despite literature suggesting that the name *Pennularia* may be an earlier name for species that are now accepted as *Collosporidium* (Butler, 1992). Some authors believe that the main reason for keeping the name *Collosporidium* is because many well-known diseases had already been attributed to

species of *Colletotrichum* by the time the genus was unified (Barnett, 1988; Dade, 1991).

The debate on classification and nomenclature of fungi identified as *Colletotrichum* has often been contentious (Dadey and Jeger, 1982; Barnett, 1988; Finsky et al., 2000). Many monogenic and nonmonogenic species have been described as or assigned to *Colletotrichum* (Gutson, 1982). A reorganization by Cohen and von Arx resulted in a large number of species having their names changed from *Peridermium* and *Glomerium* to *Colletotrichum* with *Colletotrichum glomeratum* being designated as the anamorphic state of all fungi with the teleomorph as *Glomerella elongata* (Guterson) Spald. and B. Schrenk (von Arx, 1971, cited by Sutton, 1992). *Colletotrichum glomeratum* was proposed after merging the name of the sexual stage of anamorphs of certain *Peridermium* (Poung, 1987) used by Barnett, 1988).

Species of *Colletotrichum* traditionally has been based on the shape and size of conidia and appressoria, production of perithecia or ascia, and colony growth characteristics (Gosnell and Gubler, 1990; Bensch and Bensch, 1990; Sutton, 1992). Species within the genus are generally characterized by formation of subglobose or prolate conidia. The different species may or may not produce dark brown to black sclerotia. Dark sclera often are found in the sclerotic canaliculi produced from phialides or, especially elongated and hyaline with pointed or rounded ends (Gutson, 1982). Fungal growth rate has also been proposed as differentiating species of *Colletotrichum* (Gosnell and Gubler, 1990). However, *Colletotrichum* spp. are highly variable and differentiation using morphological or growth rate characteristics may be unreliable (Chakrabarty et al., 1987; Pannan et al., 1990; Sutton, 1992). Frequency discrepancies of morphological or

phenotypic characters have been based on observations made in vitro under conditions that have rarely been standardized. This increases the uncertainty of identification especially considering the phenotypic plasticity of individual isolates (Sutton, 1983).

The usual system used for identification of *Colletotrichum* species is further compromised by the fact that many descriptions within the genus were based on host-related criteria (i.e., host spot diseases or host isolations) rather than morphological characters (Cannon et al., 2000; Desjardis and Smiley, 1988; Sutton, 1983). In our study, an improved description of *C. fragariae* and *C. glaucosporium* affecting strawberry was pursued by studying the pathogenicity and virulence of isolates of *C. glaucosporium*, *C. fragariae*, *C. trifolii*, *C. coccicola*, and *C. dematium* (Blum and Howard, 1988). After testing isolates of these pathogens on isolates and fruits of strawberry and apple it was suggested that isolates of *C. fragariae* may represent a distinct group from those of *C. glaucosporium*. However, it was also concluded that under favorable environmental conditions isolates of *C. coccicola* and *C. glaucosporium* could be as virulent as *C. fragariae* on strawberry isolates (Maas and Howard, 1983) indicating the potential for misidentification of isolates based on host reaction. Similarly, the practice of creating ecological implications in the use of the term *anthracnose* has resulted in morphologically indistinguishable *Colletotrichum* isolates being assigned to different species based on the host of origin (Sreenivasulu et al., 1996a) even though the initial source of inoculum for diseases related to *C. glaucosporium*, *C. dematium*, *C. aculeatum*, and *C. graminicola* have originated from an apparently random collection of alternative hosts from different plant species, genera and families (Smiley and Jeger, 1983; Sreenivasulu et al., 1992; Proctor et al., 1998; Madden, 1977; Smiley et al.,

1990; Sasser 1992). These studies have led to circumstantial evidence about causes of outbreaks of *Colletotrichum* spp. have been challenged or confirmed to be true (Agrios et al., 1991; Trnka et al., 1993; Sasser, 1992). In Florida, postharvest decay of mango when stored was statistically associated with slow-growing strains of *C. gloeosporioides* infect *C. acrocarum* was identified in the soil canal region of the dam (Agrios et al., 1992; Trnka et al., 1993). These studies demonstrate the use of traditional taxonomic methods with *Colletotrichum* species has resulted in “very broad variables and non-productive” taxonomic concepts (Sasser, 1992) that have the potential to produce misidentification of pathogen responsible for *Colletotrichum* diseases.

Molecular approaches to resolve the role of the taxonomy with and within *Colletotrichum* species appear to be more appropriate for inferring true phylogenetic relationships at different taxon level, and to test new and old hypotheses such as host or geographic specificity (Jin et al., 1994; Friesen et al., 2000; Sasser-Supersat et al., 1995). Molecular techniques including restriction fragment length polymorphism (RFLP) and random amplified polymorphic DNA (RAPD) analyses, were used to differentiate mitochondrial DNA (mtDNA) from European and North American isolates of *C. acrocarum*, *C. fragariae* and *C. gloeosporioides* from strawberry (Sasser-Supersat et al., 1992). Isolates of *C. acrocarum* could be separated according to geographic origin even when considerable homogeneity in mtDNA polymorphism was detected within isolates from diverse hosts (Sasser-Supersat et al., 1992). Analysis of arbitrarily primed PCR (ap-PCR) and A + T rich DNA binding proteins confirmed that grouping of *C. acrocarum*, *C. fragariae* and *C. gloeosporioides* isolates based on molecular markers could differ from groupings based on classical taxonomic characteristics (Friesen et al.,

(1911). Host specificity of *C. glaucosporoides* was evaluated by looking at the geographic relationship among isolates infecting avocado, banana, mango, and papaya (Rodríguez et al., 1992). Isolates constructed with ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) from *Glomeromyces coffeibergensis* and *C. glaucosporoides* were used as generic RFLPs and RAPD markers and to analyze DNA polymorphisms. Isolates obtained from different hosts never had the same rDNA or mtDNA RFLP patterns but isolates could not be grouped according to host with the exception of isolates from mango.

The ability to characterize isolates of fungi using molecular markers has had a profound impact on the study of several populations of plant pathogens (Brenan et al., 1991). Molecular analysis by pulsed field gel electrophoresis, RFLP, polymerase chain reaction (PCR), restriction enzyme digest analysis, sequence analysis, and nucleic acid hybridization allow a determination of dissimilarity among Δ distances between species or isolates, genome complexity, recombination, frequency, heterozygosity, gene identification of specific genes of interest, and provides a insight into the dynamics of populations (Brenan et al., 1991; Rodriguez and Rodman, 1991). In Citrus spp., analysis of RFLPs and genetic fingerprints with microzymes of various polygalacturonases, protein markers, and protein lysates, indicated that a unique sub population of *C. glaucosporoides* was the causal agent of citrus greening fruit drop (Clement and Davis, 1991; Lyseng et al., 1982; Lyseng et al., 1983). Isolation of the isolates as *C. glaucosporoides* was determined using only host-origins and morphological characters. Subsequent studies (Brenan et al., 1996) found that DNA from three isolates yielded a 460-bp fragment specific to *C. acetabulariae* when amplified by PCR with primers Cx1 and Cx2 (4 base, 50

specific amplification products were produced when using DNA from isolates of *C. glaucopernix*. These results suggested that previously described subpopulation of *C. glaucopernix* was actually *C. roseus* (Brown et al., 1996).

Construction of species-specific primers, especially from within the ribosomal DNA internal transcribed spacer (ITS) region, has been proposed as the most efficient and reliable system for detection and differentiation of *Colletotrichum* spp. (Greenen et al., 2000; Seneviratneperera et al., 1996a). However, sequence data does not always reveal significant differences between species. Sequences of a region of the internal transcribed spacer (ITS) I region of the DNA were compared between isolates of *C. glaucopernix* and *C. fragariae* from strawberry (Seneviratneperera et al., 1992). The analysis detected too few differences to provide sufficient data to separate *C. glaucopernix* and *C. fragariae* isolates based on sequence identity (Seneviratneperera et al., 1996a). However, *C. fragariae* has been distinguished as a separate species from *C. glaucopernix* and has been recognized as a teleomorph form using morphological and ribosomal DNA RFLPs, or microarray techniques (Hosaka et al., 1999; Raddad et al., 1999; Freeman et al., 1997).

Glomerella cingulata, the Sexual Stage

With few exceptions, the production of the sexual stage by species of *Colletotrichum* is rare or has not been reported (Jalton, 1992). When possible an exception has been described, mycelium production is observed usually only after the death of the host or under laboratory conditions (Akopyants et al., 1996; Hawthornth et al., 1996; Saito, 1992). Therefore, most species described as the genus *Glomerella* are associated with diseases in which a majority of the infections are usually produced by

cellules linked to anastomosis, most of which are in the Callus-nucleus zone (Sutton, 1992).

Gloeosporium vagabundum is the type species (holotype) upon which the genus *Gloeosporium* is based (Sutton, 1992). *Gloeosporium vagabundum* produces perithecia described as globose to obpyriform, dark brown to black, 12 to 200 µm in diameter, subglobose on outside, with the outside slightly papillose and a canal that is lined with paraphyses. Sometimes a poorly developed stroma can be present in the perithecia, but not the base shows groups of paraphyses mixed with asci. The anastomosis area usually contains eight conical asci, which are less than 30 µm long, mostly slightly curved, hyaline or faintly brown, and narrowly oval to cylindrical to fusiform (Hendrix, 1990; Moudon, 1970). *Gloeosporium vagabundum*, like other species in the genus, differs from other *Peritheciaceae* by the character of its stroma (Adamopoulos et al., 1990; Sutton, 1992).

In apple, *Gloeosporium vagabundum* produces latent rot of apple, which can remain dormant for years when newly mature fruit show disease symptoms (Sutton and Simon, 1981). Symptoms of the disease include formation of small, dark areas that enlarge rapidly and become circular and sunken in the center. Lesions can eventually produce rings, but with masses of pink-colored spores until the host tissue collapses and the lesion becomes dark brown to black, wrinkled, and sunken (Agrios, 1997). Apple scabs with similar symptoms have been found to produce perithecia of *G. vagabundum* under field conditions (Stone and Sutton, 1981) and non-optimal conditions have an effect on spore discharge (Stone and Sutton, 1981). High air moisture, rain, and wind can help in dispersal; ascospores released from lesions found on dead wood (Sutton and Simon, 1982). In such cases the ascospores are heavily discharged during and after rainfall, and are a significant

onset of primary oscillations when they are not subjected to wind stress (Björnsen and Røed, 1991, Røed and Björnsen, 1992). Disruptive discharge may also occur without marked net releases of G conjugate measured with a 12 hour light/dark photoperiod at 20 °C and 100% relative humidity can produce regular cycles of disruptive discharge 20 minutes after the start of the dark period (Tully and Røed, 1971, cited by Björnsen, 1992).

The ability to produce various postures under natural and artificial environmental stress is greatly among and within species of *Collembola* (Björnsen et al., 1992, Cannon et al., 2000). *Gammarella stagnalis* is basically hemispherical (self-burial) but in culture is also capable of producing stress-like variants (Alexopoulos et al., 1996, Björnsen et al., 1992). Additionally, there are observations in which hemispherical stress of G conjugate have occurred to self-burial stress during recovery (Goffin, 1996). The biology and molecular basis of many of these observations is not completely understood but it is clear that environmental oscillations can affect pathologic formation (Alexopoulos et al., 1996). In fact, stress tolerance of *Collembola* species have been reported only after adjustments in the environment were made (Alexopoulos et al., 1996, Björnsen et al., 1992, Røed, 1992) made in the case with *Gammarella graminicola*. There are no reports of G graminicola showing disruptive stress under natural conditions (Björnsen et al., 1992, Sørensen, 1992). *Gammarella graminicola* was first reported when 25-64 cm² pieces of detached moss leaves were inoculated with mycelium derived from a single parental isolate or from pooling of individual isolates of G graminicola (Peters, 1973). Pathogenic was observed when pieces of detached moss leaf were inoculated with parent strains and incubated in the moss piece of plant tissue under high moisture and continuous light

for 15-20 days at 18-20 °C (Williams and Hesse, 1991). Successful induction of sexual stages of different *Collostridium* species has also been achieved using semi-synthetic and synthetic media (Bryson et al., 1992). To test for positional production of *Collostridium* spp., it has been suggested that large numbers of isolates should be evaluated singly and in combination under different environmental conditions (Glynn et al., 1992; Jones, 1992).

In general terms, flagell cells used to suggest that they are genetically different in order to mate according to genetically determined mechanisms described as self-fertile (homothallic) or self-sterile (heterothallic) (Alexopoulos et al., 1994). In the case of *Chlamydomonas* spp. the sexual mating system does not fit into either of these broad categories of homothallic and heterothallic species. Early work, summarized by Bryson et al. (Bryson et al., 1992), suggested a heterothallic system controlled by two genes linked to formation and distribution of flagging structures in *C. reinhardtii*. One of the genes named *A*, with alleles *A⁺* and *A⁻* determines production of pedicels (*A⁺*) or none (*A⁻*). A second, *B* gene regulated the distribution or attachment pattern of these structures as changed (*B⁺*) or ventral pattern (*B⁻*) (with alleles *B⁺* and *B⁻*). In addition to the effect on the formation and distribution of flagging structures, these genes were also found to affect mating of cultured strains of *C. reinhardtii* suggesting their role as mating type genes of homothallic flag. However, homothallism was also noted when crosses between isolates with genotypes *A⁺B⁺* and *A⁺B⁻* as well as isolates with genotypes *A⁻B⁺* were crossed with isolates *A⁻B⁻* or *A⁺B⁻* and fertile microcaps were produced with each combination of genotypes. Since diploid cells homozygous to either the *A* or *B* locus, or at both loci can produce fertile microcaps under characteristics resembling homothallism

and heterodiffia-systems, the genes A and B sometimes considered part of mating type loci (Reynan et al., 1992).

These results suggested that *C. rugulosa* is a hemothallic species with the ability to behave as a heterothallic species depending on means via affecting morphogenetic pathways necessary for self-fertility (Vallascourt et al., 2000). This system fits the description of some isolates of *Colletotrichum* spp. in which otherwise heterothallic isolates, with mutations at different loci, can complement each another restoring fertility and producing self-fertile in strains of 100% (Henderson et al., 1981; Muelken, 1981 cited by Reynan et al., 1992; Vallascourt et al., 2000). This type of mating system, named relative hemothallism or unbalanced heterothallism, was first described with *Sclerotinia sclerotiorum* and has been associated, at least on one occasion, with the release of unknown *dehiscida* products in the environment (Reynan et al., 1992; Vallascourt et al., 2000).

Considerations on the reproductive biology of *Glomerella* spp. may be important in predicting whether particular genotypes may arise, or whether sexual or asexual sources of inoculum are important epidemiologically (Hidalgoson, 1996). Brown et al. (1997) recently has mentioned that genotypic variation in *Colletotrichum* populations can be generated by processes other than sexual recombination (Keller and Davis, 1997). High levels of variation among *C. glomerata* isolates from diseasiness and other hosts have been reported in several studies (Keller et al., 1993; Chakrabarty et al., 1997; Bousquet et al., 1998; Hoffman et al., 1999). Considerable diversity among isolates of *C. glomerata* in infection landing pattern in cDNA and mtDNA has been described for various infecting methods, temps, and other hosts (Keller et al., 1997). Isolates of *C. glomerata* pathogen on *Spiranthes* spp. have highly variable RAPD banding

primers (Chakraborty *et al.*, 1997). Molecular analysis using AFLP, RAPD and RAPtype polymorphism of chromosome demonstrated that occasional transfer of specific chromosomes may occur between apparently genetically distinct, isolated clonal forms of biotypes of *C. glaucosperma* producing different colonization diseases on the legumes of *Lupinus* spp. (Jehal *et al.*, 1998). In addition to actual recombination diversity in fungal populations can also arise from transposable elements and mobile temporal transposon inserts (Keller and Moss, 1992). Molecular diversity can result from accumulation of mutations and extremely long periods of independent evolution from a single source that passed the ability to infect a particular host, or by assuming that pathogenicity on a certain host may have been acquired by a large number of genetically distinct strains (Chakraborty *et al.*, 1997; Roberts *et al.*, 1992). Although these structural processes can produce variation within a species or population, natural reproduction through vegeta results in clonal population structures that have disrupted in *Botrytis*, such as widespread occurrence of identical genotypes and correlations between independent sets of genetic markers (Majumder, 1994). In many instances resistance to genetic recombination such as mycelial or vegetative incompatibility exists in several pathogen populations producing clonal population structures (Mead *et al.*, 1994).

Colletotrichum spp. as biocontrol in Fusarium

Two important diseases of sorghum caused by *Colletotrichum* have apparently reduced the role of *Colletotrichum* as a biocontrol. *Colletotrichum* species can also cause diseases on roots (pedicels, nodules, flowers (flower blight), panicles (head rot) and leaves (black leaf spot and irregular leaf spot) (Dasgupta and Bhattachary, 1993; Freeman and Khan, 1977; Howard *et al.*, 1993; Mann, 1989). In some locations, up to 80% of

transplants have been infected with *Colletotrichum* spp. prior to planting (Woodward, 1997; Brooks, 1992; Howard et al., 1992). In the U.S., yield losses greater than 50 % have been recorded when warm and humid conditions occur and serious outbreaks of fruit rot occur not developed (Howard et al., 1992; Wilson et al., 1992). Occasionally, up to 80% of the plants within a field have been infected (Brooks, 1992; Howard et al., 1992). Symptoms of anthracnose fruit rot include the formation of typical anthracnose symptoms such as sunken necrotic lesions with conidia produced in abundance at the surface (Brooks, 1992). In the 1980's, Smith (Smith, 1986) proposed the name anthracnose crown rot to distinguish the symptoms produced by *C. freyana* in strawberry crowns from those appearing on fruit. The name became widely accepted and was applied to all diseases caused by *Colletotrichum* spp. that attacked the crown. However, in many instances the symptoms are not produced by the originally described pathogen, *C. freyana*, and these symptoms are not typical of anthracnose diseases. Instead the *Colletotrichum* crown rot affects the plant by producing sudden wilt and plant death (Rosen and Carver, 1982; Hersh and Carver, 1982). A reddish brown necrotic vascular in the vascular tissue develops in the early stages of the disease and eventually the entire crown becomes discolored and the plant dies without the development of hyphae (Rosen and Carver, 1982; Howard et al., 1992; Smith, 1986). Recently crown rot epidemics were also associated with symptoms of stunting, chlorosis, and root rot in strawberry plants infected with *C. acroton* (Frostmaier and Kania, 1987). *Colletotrichum* crown rot has been proposed (Lagerd, 2000) as more appropriate name for the crown rot diseases caused by *Colletotrichum* species.

Colletotrichum scutellum, *C. fragariae*, and *C. gloeosporioides* (teleomorph *Gloeosporium fragariae*) are the three major species causing diseases on strawberry (Shepherd, 1967; Brooks, 1982; Demoyers and Baudry, 1990; Freeman and Katan, 1992; Howard et al., 1992). Occasionally, *C. gloeosporioides* also been reported as a weak pathogen of strawberry fruit (Benda and Wright, 1973). Originally only *C. fragariae* was reported affecting strawberry runners and crowns in the United States (Brooks, 1982) but in 1937 symptoms of anthracnose in strawberry fruit produced by *Gloeosporium* sp. (now reclassified as *C. scutellum* by Demoyers (Demoyers, 1981)) were reported in Australia (Waggon, 1934, 1937). It is likely that *C. scutellum* had been in the United States before 1960 (Lopez, 2000) when a crown rot producing wilting of plants and a fruit rot caused by *C. scutellum* occurred in the southeastern United States (Smith, 1969). In the late 1960s a disease found in a shipment of strawberry fruit from Louisiana to New York was associated with the presence of *Gloeosporium* sp. (Wright et al., 1968). An amended earlier 1906 Act (now Act, 1957) used by Sutton (1962) changed the name of *Gloeosporium* to *Colletotrichum* thus opening the possibility that the organism affecting States in the late 1950s was in fact *C. scutellum*.

During the last 10 to 20 years knowledge on the epidemiology of *Colletotrichum* diseases on strawberry has improved considerably. However, several aspects of the biology of the pathogen has not been extensively studied (Howard et al., 1992; Lopez, 2000). It is known that ascidia produced in multipassive manner in ascovial and dispersed by water play an important role in epidemics of anthracnose fruit rot and especially in crown rot epidemics (Ofta, 1998). Colonies of *C. scutellum* can be asexual, dispersed up to 30 cm from its inoculum source (Ting et al., 1998). In greenhouse and

long distance dispersal of nematode seems to be a consequence of nematodes being transported by transplants, field waters, farm equipment, and birds (Blanch, 1990; Lopez, 2000). In regions with lower temperatures during the winter, nematode produced from plant debris present in the soil may initiate populations each spring (Eastburn and Gubler, 1990; Wilson et al., 1992). In California, *C. nematode* was associated to strawberry plant debris for up to 9 months in fields that were not disrupted with methyl bromide (Eastburn and Gubler, 1990) and *C. nematode* is considered as a nematode that hibernated in soil between seasons in Ohio (Wilson et al., 1992).

Colletorichus does not survive between strawberry seasons in soil (Blom and Carver, 1960), and it has been suggested that *Colletorichus* spp. associated with strawberry do not survive between seasons in soil or hibernated plant debris in Florida due to hot and wet summer conditions (Howard et al., 1992). However, this hypothesis has not been experimentally tested. Alternative hosts are a potential source of primary inoculum for *Colletorichus* spp. diseases. Symptoms found on almond, apple, peach and pear have been linked to crown rot/lesion of inflorescence produced by strains of *C. glaucogriseus* and *C. nematode* (Gubler and Berman, 1987; Berman et al., 1991). Both species are known to have extensive host ranges (Dyke and Ikadue, 1979; Martin, 1971). Transplants are the most likely source of primary inoculum (Freeman and Katan, 1990; Howard et al., 1992), and transplants infected or infested with *Colletorichus* spp. have been found in Louisiana (McIntosh et al., 1992), California (Eastburn and Gubler, 1990), and Israel (Freeman and Katan, 1987; Freeman et al., 1992). Epidemiology have also originated from other sources of inoculum (Eastburn and Gubler, 1990; Hara and Carver, 1960; Wilson et al., 1992). Blom and Carver (Blom and Carver, 1960) suggested that

monkeys might survive at lower temperatures after *C. jejuni* was isolated from monkeys stored at 27 °C over a 36-week period in Louisiana. Nevertheless, the source of primary infections for *Callosporichium* infections in Florida is still unclear (Lewandowski et al., 1982; Legard, 2000). The role of the teleomorph of *Callosporichium* spp. in virulence is believed to be unknown and its role in the disease cycle has been disregarded (Pinner et al., 1982; Mann, 1994).

Microarray and deletion of important fungal pathogens or parasite resistant strains can be estimated using information from nuclear and mitochondrial protein sequence matches and death rates, and genotype and allele frequencies and other population parameters (Anwar, 1994; Gillespie, 1998; Blackmore et al., 2004). Even in the case of species lacking known sexual stages, estimation of the likelihood of recombination can be assessed from studies of multilocus population structure (Majumdar, 1996). Evaluation of the genetic structure of populations of *Callosporichium* affecting streptococci should provide important insights into the cause of *Callosporichium* diseases, and improve knowledge about the biology of these pathogens and their related epidemics. For sexually reproducing fungi such as *C. neoformans* and *C. glabrata*, detection of subclones using population genetic approaches may provide insights on the sources of the sexual population or be an indicator of host specificity (Pinner et al., 1998; Sarmiento-Gonzalez, 1999a). PCR-based techniques have been used to screen DNA polymorphisms at a wide variety of fungi. Random amplification of polymorphic DNA (RAPD) was used to study population genetic structure and host specificity of *Fluoribacterium* *arquiporum* (Carrubba et al., 1992) and *Microascus* spp. (Pinner et al., 1999). Differentiation between isolates of *C. glabrata* from LB and human blood

populations was reached using selectively pruned PCR (sp-PCR) analysis (Friesen et al., 1996). These techniques (sp-PCR and RAPD) require small (ng) quantities of DNA, are less applicable to many cultures, do not involve extensive reagents, most of the amplified loci are randomly associated and arise from multiple isolated regions of the genome (Haleidi et al., 1995; Friesen and Milgroom, 1993).

Control of *Colletotrichum* diseases begins at the variety and continues in that production fields (Friesen et al., 1993; Karamak, 1999). In strategy fields, cultural management such as the use of drip-irrigation, raised bed plantations, use of resistant cultivars, and clear-glazing work, are important methods for controlling *Colletotrichum* diseases (Lopez, 2000; Miao, 1998). Because these diseases are more severe during periods of warm temperatures and high rainfall (spots are quickly dispersely spread) of conditions, be reduced by avoiding excessive use of overhead irrigation during plant establishment and flower periods (Lopez, 2000; Yang et al., 1998). Producing tomatoes in tunnels might be an alternative for controlling *Colletotrichum* diseases. The advantage of growing in tunnels is better control of environmental conditions, which helps to increase the efficacy of fungicides and prevent the development of environmental conditions favorable for disease (Lopez, 2000).

Resistant cultivars are an excellent means for controlling diseases (Lopez, 1997; Gadway, 1993; Miao, 1993). However, commercial greenhouse cultivars vary in their susceptibility to *Colletotrichum* diseases (Dourado-Neto et al., 1997; Ogawa and Smith, 1991; Smith and Black, 1987). Resistance to anthracnose fruit rot and *Colletotrichum* crown rot can be influenced by environmental conditions (Dourado-Neto et al., 1998; Smith and Black, 1987) and the susceptibility of cultivars is also likely to vary depending

on the different pathogens they are exposed to in the field (Agrios, 1997; Douglas-Scott, 1997; Caplan and Bough, 1998).

The best way to manage diseases is to prevent the introduction of the pathogen to the crop (Agrios, 1997; Miley, 1992) and the use of pathogen-free planting material must be a priority to avoid sources of primary inoculum for disease epidemics (Hennrich et al., 1991; Miley, 1992). However, there are no reliable methods for detecting asymptomatic free of *C. necrotorum* and *C. glaucosporus* (Lepoint, 1998). Strawberry plant tissue tested with peroxidase (H 51-H 11 serum reagents) or exposed to freezing temperatures (-1°C) has shown potential for the detection of latent infection of *Colletotrichum* spp., thus allowing new alternatives for disease certification programs (Gardner and Pinner, 1994; Miley and Lepoint, 1995). Until then, special care must be taken to prevent the occurrence of disease epidemics or to not manage them correctly with good management of diseases (Collins and Broughton, 1998; Finnie et al., 1990; Miley, 1992).

Soil fumigation with methyl bromide and chloropiquet (30 L at 100 kg ha⁻¹/year to planting 114 days or more before crop planting) is a successful method for managing many diseases of strawberry (Miles, 1996; Miley, 2000). Soil fumigation helps to destroy potential inoculum in soil or plant debris (Miles, 1996). However, the use of methyl bromide is being stimulated due to environmental concerns (Miley, 2000). Alternatives to methyl bromide such as vapour, bromine, and chloropiquet have yielded promising results (Finnigan et al., 1991; Hansen, 1994; Miley, 2000). However, more research and growth are not conclusive about the actual alternatives to methyl bromide (Jensen, 1994).

Protective fungicides applied from flower bud emergence through harvest are widely used in strawberry production (Miles, 1994). However, currently labeled fungicides have only limited efficacy managing *Colletotrichum* diseases in strawberry (Legard, 2008; Miles, 1994). Benlate and captan are frequently applied in strawberry fields (Legard, 2008) to protect the new tissue against many diseases, particularly when rain is predicted and the potential increases for sporulation and dissemination of spores in the field (Flegrand et al., 1985). However, resistance to benlate¹ in *Colletotrichum* spp. has been well documented (Lalancette, 1993; Smith and Black, 1994). Captan also fails to provide complete control of *Colletotrichum* diseases (Freeman et al., 1987; Howard et al., 1992; Legard, 2008). However, recent reports indicate that captan used on a 3 days spray schedule can result in significant control of anthracnose fruit rot produced by *C. acutatum* in comparison with an untreated control (Legard et al., 2006). Azoxystrobin (Quadris) is another fungicide that controls anthracnose fruit rot caused by *C. acutatum* (Legard and Chandler, 2007) and with prochloraz, 75% or prochloraz 2x may provide alternative to control of *Colletotrichum* diseases in the near future (Freeman et al., 1987). However, when conditions are favorable for disease development the use of chemicals may be of limited help for management of *Colletotrichum* diseases (Freeman et al., 1987; Howard et al., 1992; Legard, 2008).

Anthracnose fruit rot and *Colletotrichum* crown rot cause serious reduction in marketable yield and presents significant challenges for plant certification programs and movement of strawberry material worldwide (Flegrand, 1987). The pathogens responsible for these diseases can also cause on all parts of the plant, including flowers, buds, leaves, and roots (Freeman and Kania, 1987). However, it is clear that many

significant ecological and systematical questions need to be resolved about these diseases before a more effective management of *Colletotrichum* diseases in strawberry can be implemented. Consideration of the biology of *Colletotrichum* spp. will be important in predicting the nature of primary inoculum for *Colletotrichum* epidemics in Florida, and sustainable approaches may be used to manage issues involving the uncertainty in taxonomy of *Colletotrichum* species or in assessing the likelihood of recombination by evaluating the genetic structure of populations of *Colletotrichum* species infecting strawberry.

CHAPTER 3 OVERWINTER SURVIVAL FOR POTENTIAL INOCULA OF COLLECTED ORCHARD CROWNS NOT IN BURIED STRAWBERRY CROWNS

Introduction

Strawberries are grown as an annual winter crop in west central Florida. Coccin-
top transplants from nurseries in the southwestern U.S. or southwestern Canada, are set
down from September through early November in sandy/beneath-limegravel plastic-
mulched beds. Fruit are harvested from late November through early April (Harris and
Henderson, 1987). At the end of the season, growers destroy the plants by applying
picopact or mowing the tops off the plants. Some growers will plant spring vegetable
crops over the mowing beds after killing the strawberry plants. From April to late May a
grain or legume cover crop is typically planted. During August to early September the
cover crop is incorporated and raised beds are formed and fertilized with a mixture of
mulched limeade and chileanpierre. In Florida, only the beds are fumigated and
newberries are grown in the same location every season without crop rotation.

Colletotrichum spp. cause a wide range of diseases of strawberry, and lesions of
leaves, stems, and roots can result in serious yield losses (Dougherty and Dandry, 1955;
Freeman and Kania, 1960; Hara et al., 1971; Hara and Carter, 1980). Many of these
diseases have been called enhancement lesions of the production of typical symptoms
which include limited necrotic lesions and hyphae (Stewart and Allington, 1984;
Smith, 1980). Plants with symptoms of *Colletotrichum* infection are not suitable as isolates without

the crown plant. Within the crown, reddish brown necrotic streaks develop in the vascular tissue (Smith, 1987), and eventually the entire crown becomes discolored and the plant dies (Hart and Carver, 1982). In Florida, crowns are typically killed 2 to 4% of plants each season (Legend, unpublished data), but losses of up to 90% within individual fields have occurred when conditions are conducive for serious outbreaks (Howard et al., 1992). *Colletotrichum rotatum*, *C. fragariae*, and *C. gloeosporioides* (anamorph *Gloeospora fragariae*) are the three major *Colletotrichum* species that have been reported to cause crown rot in strawberry (Boshard, 1987; Boscha, 1982; Freeman et al., 1991; Hart and Carver, 1982; Howard et al., 1992; Smith, 1986). Recent *Colletotrichum* crown rot epidemics in Florida have been predominantly caused by *C. gloeosporioides* (Legend et al., 1992).

The source of primary inoculum for epidemics of *Colletotrichum* crown rot of strawberry in Florida is unclear. Transplants are the most likely source of primary inoculum (Freeman and Kania, 1990; Howard et al., 1992), and transplants infected or infested with *Colletotrichum* spp. have been found in Louisiana (Widness et al., 1992), California (Eastburn and Gubler, 1991), and Israel (Freeman and Kania, 1990). Epidemics have also originated from other sources of inoculum (Eastburn and Gubler, 1991; Hart and Carver, 1982; Wilson et al., 1992). In California, *C. rotatum* overwintered in strawberry plant debris for up to 3 months in fields that were not fungicide with methyl benlate (Eastburn and Gubler, 1990), and *C. rotatum* overwintered in mummified fruit/blossoms and between seasons in Ohio (Wilson et al., 1992). Hart and Carver (Hart and Carver, 1982) suggested that inoculum might survive in crown tissue after *C. fragariae* was isolated from crown rot at 5°C over a 30 week

period in Louisiana. It has been suggested that *Colletotrichum* spp. associated with secondary rice rust likely to survive because of rice or related plant debris in Florida due to hot and wet summer conditions (Howard et al., 1992). However, this hypothesis has not been experimentally tested. The purpose of this study was to evaluate the ability of *C. glomerosporium* to overwinter in crown roots under field conditions in Florida.

Materials and Methods

The overwinter survival of *C. glomerosporium* was evaluated under field conditions during 1998 and 1999 in the University of Florida Gulf Coast Research and Education Center (GCREC), Stone, Louisiana grown grain-rice transplants of *Calasencia* variety used both years. Plants were set into raised plastic-mulched beds in October each year and grown and harvested through April. Plants used in 1998 were planted at non-damaged soil. For the 1999 experiment, plants were set in a field that had been treated with methyl benzoate and chlorpyrifos (40:1 at 100 kg/ha) prior to planting. No other fungicides were applied either season. Other pesticides were applied as needed to control arthropod pests. Severe *Colletotrichum* crown rot symptoms developed in the plots at the end of each growing season. In June of both years, 4 months after planting the field, plants without visible symptoms of *Colletotrichum* crown rot were selected from up-rows with severe crown rot (50 or 33% of plants with crown rot symptoms). One or two weeks before collecting plants for the burial studies, 10 to 20 asymptomatic plants were tagged by placing detached crown tissue into a *Colletotrichum isolatus* medium (CIM) modified from Steiner and Watson (Steiner and Watson, 1963). The medium contained 6% of D-threo-potato-dextrose broth, 14 g of

Dilco agar, 150 mg of ampicillin, 150 mg of streptomycin sulfate, 3 mg of gentamicin (Pharmacia and Kanto, 1987), 180 µl of Tergitol, and de-aerated water to 1 liter. *Cellulomonas* spp. were detected in most of the sampled systems.

The plot used for the 1998 burial study had been cropped with blackberry during the previous 4 years; but the stand had been removed and the area converted to fallow 12 months prior to this study. The area was subplanted 24 hr prior to burial of the systems. On June 4, 80 plants were dug from two adjacent beds (harvest beds) and transplanted close to the primary crown of leaves, roots, and secondary crowns. Two primary crowns were placed inside a porous cloth (Mire cloth, Calhounham, Co., La Jolla, CA) bag with 20 cc of water-dispersed seed and buried at a depth of 11 cm in a different location from being. Burial locations for the bags were organized in a randomized complete block design with four replicates and sampling time as the treatment. At each sampling time (0, 3, 14, 20, 42, 58, 70, 86, 118, and 140 days after burial) one bag was excavated from each of the four replicated plots (eight systems total). To ensure the study of *C. phaeosporus* in the root in the burial systems, systems from one plot (one CIM Transplant segment approximately 1 cm thick) were cut from the upper and lower portion of the crown and from two segments within the middle portion subject surface sterilized scalpel. The segments were surface-sterilized for three minutes in a solution of 0.2% sodium hypochlorite, then rinsed for 3 minutes in sterile de-aerated water. The segments were air-dried on sterile paper towels, cut as before and placed on CIM medium. All cultures were incubated for 3 to 5 days at 28°C with continuous fluorescent light (Philips Universal, 750 lumens, 4 600 B).

For the 1999 study, the burial field had been cropped in strawberry the previous season using conventional practices, including fertilization with methyl benzoate. The area was harvested 24 hr prior to burial of the crowns. On 10 July 2001 strawberry plants were collected from a field that had been treated with methyl benzoate and chlorpyrifos (CM 2 at 100 kg/ha) prior to planting (Barrow field) and treated as described for the 1998 experiment. Individual crowns were placed inside black cloth bags with 50 cc of soil from the burial row. One bag was buried at each of two depths, 5 cm and 10 cm at the same location, comprising a single sample pair for each time period. Treatments were replicated 10 times and arranged in a complete block design. Pairs of crowns were recovered from 10 burial locations in each sampling time (0, 7, 14, 21, 28, 35, 42, 49, 56, 70, 90, 110, and 140 days after burial). After recovery, crowns were processed as in the 1998 study. In addition, crown leaves containing chlorophyll contents in CM4 medium, was occasionally not used 3 to 4 mm pieces of finely ground with an electric coffee grinder (Brewer Inc. Woburn, MA) for 10 seconds. The macerate was surface sterilized with 80% ethanol and not dried between samples. The ground tissue was soaked with 20-ml of sterile deionized water and diluted 10 fold during the first six weeks of the experiment and 1 liter after that, then 100µL of the suspension was plated onto CM4 medium. Inoculum plates were incubated at 24°C for 3 to 5 days under continuous fluorescent light (Philips General T58 lumens, 4000 K).

Inoculum cultures were evaluated for the presence of *Colletotrichum* spp. using a dissecting microscope and identified based on colony growth rate, coloration, and shape. After 3 to 5 days of incubation, colonies of *Colletotrichum* spp. were flat growing, white or orange, with or without dark centers and smooth borders at the edge of the colonies.

Slant cultures were grown on potato dextrose agar (PDA) and then sporulated microscopically based on spore morphology. *Colletotrichum gloeosporioides* isolates had cylindrical shaped spores and *C. acutatum* isolates had fusiform shaped spores (Smith and Black, 1990). Microscopical cultures of representative isolates were further characterized by polymerase chain reaction (PCR) amplification of the ribosomal DNA internal transcribed spacer (ITS) region (Greenwood et al., 1996a, 1996b). Conical suspensions produced from cultures grown on PDA were used to inoculate 150 ml flasks containing 100 ml of sterile linumseed medium (9 g of linum yeast extract, 15 g linum soluble meal, 1 g of glucose potassium phosphate, 0.5 g of magnesium sulfate, and 1 liter of distilled water). Mycelia were grown for 4 or 5 days on a shaker at 150 rpm. Mycelia were then rinsed with sterile distilled water and freeze dried using a vacuum liquid evaporator (Jasco, Inc., Winchester, VA). DNA extraction was performed using 200 mg of dried mycelium and a Fast DNA spin kit (catalog # K050-200, Bio 101, Inc., Vista, CA). PCR amplifications were carried out in 30 μ l reactions containing 2 μ l of DNA, 2 μ l of 10 X reaction buffer (500 mM Tris at pH 8.5, 2.5 mg of BSA/ml, 30 mM MgCl₂, 5% PEGol 400, 10 mM Dithiothreitol, Kitho Technology, Inc., 1778 Redeli Drive, 1 μ l of 1.5 mM MgCl₂, 2.8 μ l of dNTP (10 mM P/L biotechnology), 2.2 μ l of taq polymerase (1 unit/ μ l) and 10.8 μ l of sterile distilled water. One μ l each of the universal internal primer from the ITS4 region (5'-TCTCCGCTTATTGATATGC-3') and the species-specific primer Colad (5'-GGGAAGGCTCTCGGGG-3', 10 μ M) for *C. acutatum* or the species primer Cglf (5'-GAGGCTCCCGGGGCGGGGGG-3', 10 μ M) for *C. gloeosporioides* were used for identification (Greenwood et al., 1996a, 1996b).

The pathogenicity of *Colletotrichum* spp. from lateral stems was evaluated in greenhouse experiments. Twenty isolates (7% of the isolates recovered) were randomly selected among 451 isolates collected during the 1999 study. Seventeen of the isolates were *C. gloeosporioides* and three *C. coccum*. Monocotyledonous isolates were grown on PDA for 3 days at 25°C with continuous fluorescent light. For each treatment, needles were harvested and debarked in sterile distilled water to 1×10^6 conidia/ml. Three strawberry plants of cultivar Camarosa with average ca. 0.3 cm in diameter, were inoculated with 1 µl isolate suspension used to wound and inject 0.4 ml of wound suspension in leaf axils in the crown area. After inoculation, plants were incubated at 24 to 25°C for 48 hr in a humidity chamber and translocated in a greenhouse at the same temperature for 4 weeks. One strawberry isolate of *C. coccum* from an inflorescence from 1999 and one of *C. gloeosporioides* isolates from a diseased crown (*Colletotrichum coccum* only) were also included in the test, and sterile distilled water was used as control. Plants were evaluated weekly for symptoms of disease, which included wilting and plant collapse. The experiment was repeated twice. An isolate was considered to be pathogenic if two of the three inoculated plants wilted and collapsed within 30 days of inoculation. Crown segments from diseased plants were surface sterilized, incubated and plated onto CTM for isolation and confirmation of the pathogen. Weather parameters during field and greenhouse experiments were recorded using dataloggers.

Results

At the time of burial in 1998 (July 4), *C. gloeosporioides* was isolated from surface sterilized crown segments from 87% of the crowns (Fig. 1a). Over the next three

recovery of larval site pathogens was collected from 100% of the crickets. Recovery of *C. glabrata* began decreasing 42 days after burial when *C. glabrata* was recovered from 75% of the crickets. After 56 days of burial the pathogen was not recovered from any of the samples.

In 1999, after segments were surface monitored and placed, *C. glabrata* was recovered from 75% of the crickets in the time of burial (July 11) at both 3- and 13 cm (Fig. 1A). After 7 days of burial, the recovery of *C. glabrata* increased to 80% and 80% at 3 and 13 cm, respectively. Recovery of *C. glabrata* increased to 100%, 14 days after burial at 13 cm and 21 days after burial at 3 cm. Recovery of *C. glabrata* from buried crickets then declined to 90% at 13 cm 28 days after burial and 80% at 3 cm 28 days after burial. Forty-two days after burial, the pathogen was recovered from 60% of the crickets at each depth. Fifty-one days after burial, *C. glabrata* was recovered in 20% of the crickets at 3 cm and 30% of crickets at 13 cm. Twenty days after burial, only 10% of the crickets from both depths yielded *C. glabrata*. *Colletotrichum glabrata* was not recovered on crickets at either depth after 56 days or more.

Recovery of *C. glabrata* from ground crickets was on dilution plates (Fig. 1B) was similar to recovery from crickets segments. *Colletotrichum glabrata* was recovered from 70 and 80% of the crickets placed at 3- or 13-cm deep, respectively, at the time of burial. The recovery of *C. glabrata* increased to 100% at both depths after 11 days, declined to 20 to 40% after 49 days, and further declined to 10 to 20% after 70 days. The fungus was not recovered from crickets buried for 98 days or more. When the data are from both plating methods were combined, the recovery of *C. glabrata*

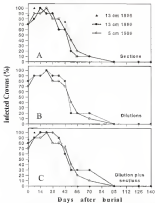


Figure 1. Frequency of recovery of *Collostrachan glaucoparvulus* from buried starfish spines. (A) Recovery from surface material: crowns, segments buried at 13 cm in 1999 and 5 and 13 cm in 1998. (B) Recovery by dilution plating of ground tissue: crowns buried at 5 and 13 cm in 1999. (C) Percentages of crowns on which *C. glaucoparvulus* was detected from either surface sterilized segments or dilution plating of ground tissue.

from buried-cornets was improved in comparison to either method alone (Fig. 1C). The increased recovery of *C. glabrata* was due to detection of the fungus in some of the cornets with only one or the other plating method. Specifically, at sample dates 35, 54, and 74 days after burial, *C. glabrata* recovery in CS was improved from 10% to 30% to 50% recovery of the pathogen when data from both methods were combined.

Colletotrichum cocciniae was also recovered from buried cornets, but only in 1998. However, *C. glabrata* was the predominant *Colletotrichum* species recovered throughout the experiment. The recovery of *C. cocciniae* was similar in both burial depths (Kivile-Piddis, unpublished data), and was recovered from 60% of buried cornets after 7 and 14 days of burial. The recovery of *C. cocciniae* from buried cornets declined to 30% after 28 days, and was not recovered from buried cornets after 54 days or later of burial.

Weekly average precipitation was similar during the experiment in 1998 and 1999 (Fig. 2A). The average weekly precipitation during the burial period was 3.9 mm in 1998 and 3.1 mm in 1999 (3.4 mm thirty-year average). During the first 30 days of the burial period, precipitation was greater in 1998 than 1999 (7.3 mm vs. 4.5 mm weekly average precipitation). However, from August through September (approximately 65 to 115 days after burial) average weekly precipitation was higher in 1998 (16.4 mm) than in 1999 (3.9 mm).

Mean weekly air temperatures from June to October were higher in 1998 than in 1999 (27.2° C versus 16.4° C) and higher than the thirty-year average (23.2° C). Higher temperatures were especially prevalent during the first 23 days of burial in 1998 when

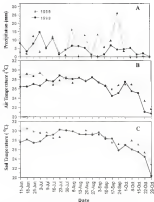


Figure 2. Weekly precipitation (A), weekly average air temperature (B), and weekly soil temperature (C) at the Gulf Coast Research and Education Center in Davie, Florida during the summer and fall of 1998 and 1999. No soil temperature data was recorded between 23 July and 6 August 1998.

comparisons 1995 (Fig. 2B). Average weekly soil temperatures were 2° or 3° C higher than air temperatures during 1994 and 1995 (Fig. 2B, C). Average weekly soil temperatures were higher during the first 50 days of the burial period in 1994 than 1995 (Fig. 2C). Soil temperatures were not recorded between July 25 and August 16, 1994 due to equipment malfunction. Daily soil moisture content (water potential) was recorded in 1995 (Fig. 3). The lowest water potential (-4.033 MPa) occurred 45 days after burial and corresponded with the maximum average soil temperatures (35.3° C), highest air temperatures (35° C), and reduced precipitation (2 mm in 1995) (Figs. 2 and 3).

Fourteen of 17 *C. gloeosporioides* isolates from buried crowns were pathogenic and produced typical crown rot symptoms on strawberry (*v.* plant wilting and death). Three isolates of *C. gloeosporioides* and all three crown rot isolates of *C. acutatum* evaluated were not pathogenic. Crowns of plants inoculated with pathogenic isolates exhibited abundant necrosis and rotting from strobiles. Plants inoculated with *C. gloeosporioides* isolated from a diseased crown, developed typical crown rot symptoms whereas the *C. acutatum* isolates from diseased fruit did not produce *Colletotrichum* crown rot symptoms. No symptoms of *Colletotrichum* crown rot developed under similar water infiltration conditions.

Discussion

Colletotrichum gloeosporioides did not prove to be as lethal to buried strawberry crowns long enough to be a major source of primary inoculum for *Colletotrichum* crown rot epidemics in fruit production fields in Florida. Although *C. gloeosporioides* was frequently isolated from crowns of strawberry early in the burial period, a quickly declined to undetectable levels within 30 to 60 days of burial. Because the time period between incorporation of

strawberry debris into the soil at the end of one season, and tripling time for the subsequent season is usually 120 to 180 days, there is sufficient time for strawberry viruses to decay and residues of *C. gloeosporioides* to be eliminated.

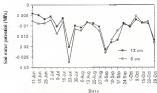


Figure 3. Average weekly soil water potential recorded at 5 and 13 cm during the summer and fall of 1999 at the Gulf Coast Research and Education Center, Dover.

Following an initial increase in the frequency of *C. gloeosporioides* during the first 3 to 4 weeks of burial, recovery of the pathogen decreased. Colonization of *gloeosporioides* was not measured from buried corms 50 days after burial in 1992 or 90 days after burial in 1999. These results suggest that *C. gloeosporioides* does not survive in buried strawberry corms long enough in Florida to be an important source of primary

wooded forages and apiculture, even though straw-burns are regulated in the state field every year. Several productive practices in Florida limit the opportunities for invasion of *C. glaucosporialis* in overwinter as plant debris or soil. Wheat can mow after the end of the season (late March or early April) plants are typically killed. Growers may remove the plant's mulch and incorporate plant material into the soil at the time or use paspalum to kill the plants, and then use the mowing beds for a spring vegetable crop. Under these circumstances, strawberry debris would not be incorporated until May after the vegetable crop is harvested. These experiments involved a worst case scenario where strawberry plants would be grown until they are incorporated at the end of a late vegetable crop in early June. In most commercial situations, the strawberry plants would have been killed 1 to 2 months earlier. After a summer cover crop of grass or legume is grown, and usually incorporated in August, the fields are harrowed with a mixture of methyl bromide-chloropicolin and beds for the next season formed in September and October. Fungicides with methyl bromide also eliminate *Callosobruchus* spp. from soil (Brooks and Gubler, 1990).

Experiments have shown temperature limits for *Callosobruchus* spp. in strawberry debris. In Louisiana, moulting of *C. fregator* was able to survive up to 7 months at masses of whole strawberry plants kept at 17°C (Hart and Carter, 1980). *Callosobruchus* *rossensis* survived from November to July in buried maize segments placed 5 to 7 cm deep in northern California (Brooks and Gubler, 1990). However, environmental conditions during the summer in most central Florida are dramatically different from the winter in Louisiana or in northern California. It is likely that the lower temperatures during the winter in Louisiana and California would restrict the survival of

Chlorosporium spp. on plant debris. It has been hypothesized that survival of *C. roseum* may improve at lower temperatures (Jurek and Gubler, 1990). This may be due to reduced colonization of plant debris by the pathogens and other soilborne microorganisms that would compete for the resources (Jurek and Gubler, 1990; Jurek and Gubler, 1990). The soil was warmer in Florida would hinder the reproduction and breakdown of strawberry plant debris. In our experiments, the path of buried crown had decomposed completely by late October and November, leaving a hollow crown with only the woody cortex remaining. This observation supports the suggestion that most of the path borne has been collected and decomposed by the end of the experiments leaving little or no substrate for survival of the pathogens.

Soil moisture content may have affected the survival of *C. glomerosporium* in buried strawberry crowns by affecting the activity of the fungi or by indirectly disrupting the activity of its competitors. Effects of soil moisture content on survival of *Chlorosporium* spp. are documented in the literature (Jurek and Gubler, 1990; Norman and Sorensen, 1997). When inoculated strawberry petioles were buried in an air-dried soil or flooded soil for 7 weeks at 25° C, *C. roseum* was detected in 60% and 0% of the petioles, respectively (Jurek and Gubler, 1992). In Florida, the survival of *C. roseum* in infected plant debris of individual form or in soil increased with reduction in soil moisture (Norman and Sorensen, 1997). Although the effect of various soil moisture and temperature on the survival *C. glomerosporium* in strawberry crowns is unclear, it is likely that it would behave similarly to *C. roseum* in plant debris or soil.

The lower precipitation recorded from August to September in 1994, in comparison to the same period in 1991, obviously reduced the moisture content of the

not during that period. This moisture may have affected the ability of *Colletotrichum* spp. to survive by influencing the activity of other organisms. It has been reported that the competitive saprophytic colonization ability of *Trichoderma harzianum* on buried plant remains was reduced when the soil was flooded or there was a dramatic reduction in the moisture content of the soil (Linderman and Butler, 1991). Traces plated onto CHA in July 1998 produced numerous colonies of *Fusarium* spp. and *Trichoderma* spp., with the later growing abundantly out of agarosols placed at the end of July (Lieber, unpublished data). In 1998, the increased mortality of these species from crown rot was not observed until late August. In addition to environmental differences between the 1998 and 1999 experiments, the spores in 1999 were grown and buried in soil that had been fungicide with methyl benodanil whereas in 1998 the sites were not fungicide. Soil fungicides affects the activity of microbes in buried soil (Whitney, 1978; Yoccoz et al., 1994). In the 1999 experiment, direct nematodes was recovered from buried crowns that in the 1998 experiment (Lieber/Pudlik, unpublished data). Thus, the extended survival period for *C. gloeosporoides* in 1999 may have been caused by differences in microbial activity due to environmental conditions or soil fungicide.

Most isolates of *C. gloeosporoides* caused *Colletotrichum*-crown rot symptoms on the germination bioassay, but none of the *C. roseum* isolates caused crown rot. These results support the observation that *Colletotrichum roseum* is a pathogen in Florida nurseries caused by *C. gloeosporoides* (Lopez et al., 1998). It is unclear why some of the *C. gloeosporoides* isolates recovered from buried crowns did not cause crown rot in the bioassay. A possible explanation is that not all isolates of *C. gloeosporoides* are causal. Some strawberry crown rot pathogens are non-burial. The plants used for the burial

plants did not have symptoms of *Colletotrichum* crown rot at the time of collection, and the detached crowns could have been released by non-pathogenic isolates of *C. gloeosporioides*. Crowns not displaying symptoms of *C. gloeosporioides* transplanted from store-bought potatoes have also tested that none of these isolates do not cause crown rot. (Lopez, unpublished data)

These results show that propagules of *C. gloeosporioides* in buried store-bought crowns are not likely to be an important source of inoculum for *Colletotrichum* crown rot epidemics in Florida. Other sources of inoculum, such as infected or infected composts or alternate hosts, may be more important in the epidemiology of *Colletotrichum* crown rot in Florida. However, due to the potential for more survival of inoculum in well-preserved store-bought isolates, it is prudent that growers use cultural practices that promote the insulation of crown tissue in the soil. More research is necessary to further elucidate sources of primary inoculum for *Colletotrichum* crown rot epidemics in Florida.

CHAPTER 4 ECOLOGICAL AND POPULATION DYNAMICS OF COLLETOSPORUM SPECIES THAT CAUSE CROWN ROT AND FRUIT ROT OF STRAWBERRY

Introduction

Anthracoenous fruit rot and *Colletotrichum* crown rot are important diseases of strawberry worldwide (Doughen and Baudry, 1995; Freeman and Kates, 1993; Howard et al., 1992; Lapsat, 1990). Symptoms of anthracnose fruit rot include the formation of rotten necrotic lesions with rotches produced in abundance in berries (Brooks, 1982). In contrast, *Colletotrichum* crown rot kills the plant by producing reddish brown necrotic streaks in the vascular tissue and plant collapse a short time development of mycelium (Lapsat, 1990). In some locations, up to 10% of transplants have been affected with *Colletotrichum* spp. (Bombaril, 1997) prior to planting, or killed by *Colletotrichum* crown rot (Howard et al., 1992). In the US, yield losses attributed to fruit rot are a loss greater than 50% when water and humid conditions occur during various outbreaks of fruit rot developed (Horn et al., 1990; Wilson et al., 1992).

Colletotrichum acroasium, *C. fragariae*, and *C. gloeosporioides* (anamorph *Gloeosporium fragulae*) are the three major species that cause diseases on strawberry (Brooks, 1982; Brooks, 1984; Doughen and Baudry, 1995; Freeman and Kates, 1993; Howard et al., 1992). The *Colletotrichum* anamorph does not have a recognized role in strawberry epidemiology and its function in the disease cycle is ignored (Howard et al., 1992; Iltis, 1990). However, *Gloeosporium* spp. do play a role in the epidemiology of

diseases affecting other ornamental and perennial crops including other members of the Rosaceae family (Sutton, 1992). On apple, white-*C. gloeosporioides* and *C. rosicola* produce a disease called black rot, necrotrophic rot produces the primary inoculum for epidemics in orchards (Jones and Sheno, 1981).

Species identification in *Colletotrichum* traditionally has been based on the shape and size of conidia, production of perithecia or setae, and colony growth characteristics (Kanehl and Gubler, 1981; Smith and Black, 1990; Sutton, 1992). However, these characteristics can be highly variable in *Colletotrichum* spp. and differentiation based on morphological characteristics may be uncertain (Chakrabarty et al., 1997; Freeman et al., 1998; Sutton, 1992). This variation has led to numerous cases of incorrect species identification (Agrios et al., 1992; Turner et al., 1994; Sutton, 1992). In California, a group of necrotic lesions from apple and peach originally identified as *C. rosicola* were later reclassified as *C. rosicola* (Kanehl and Gubler, 1981). In Florida, perithecia from a group of citrus was originally identified as slow-growing strains of *C. gloeosporioides* before species specific PCR primers revealed that *C. rosicola* was the correct identity of the pathogen (Agrios et al., 1992; Brown et al., 1994; Turner et al., 1994). Construction of species-specific primers, especially from the ribosomal DNA internal transcribed spacer (ITS) region, has been proposed as the most efficient and reliable system for detection and differentiation of *Colletotrichum* spp. (Freeman, 2000; Sivasubramanian et al., 1994a). However, strains of *C. fragariae* are still difficult to distinguish from those of *C. gloeosporioides* using morphological characters or ITS primers (Bakke, et al., 1998; Sivasubramanian, et al., 1994a). Molecular evidence from mitochondrial DNA hybridization, ribosomal ITS sequencing, and random amplification

of polyploid DPAAs (RAPDs)-based close genetic relationship between *C. pteris* and *C. glaucopetiolata* (Srinivasanprasad, et al. 1996b, Suresh 1997)

The biology and epidemiology of *Colletotrichum* spp. on strawberry are unclear (Howard et al., 1992, Leger et al. 2000). In Florida, closely produced strawberry plants are grown as an annual winter crop. Green top antraxnose, from runners in the southeastern U.S. or northeastern Canada, are planted in the fall as damaged plants-molded initial beds. Plants are typically harvested from mid-November to early April. Although the source of primary inoculum for *Colletotrichum* epidemics in Florida is uncertain (Howard et al., 1992, Leger, 2000), transplants are the most likely source (Howard et al., 1992, Freeman et al., 1997). Transplant-related with *Colletotrichum* spp. have been reported in California (Elsden and Gubler, 1990), Israel (Freeman and Kama, 1997) and Switzerland (Bosland, 1977). In temperate production regions, inoculum from plant debris may initiate epidemics each spring (Elsden and Gubler, 1990; Wilson et al., 1992). However, under southeastern United States, *Colletotrichum* does not survive between strawberry seasons on plant debris (Liu et al., 1999) as in cool (Hart and Carter, 1980).

Evaluation of the genetic structure and relationships among lineages of *Colletotrichum* spp. infecting strawberry should provide insight into their biology and the ecology of the disease. For annually reproducing fungi such as *C. verticillium* and *C. glaucopetiolata*, detection of sublineages among populations within a particular area can indicate the occurrence of sexual recombination, or seed bank or source specificity, or test assumptions about the role of the teleomorphous state in the epidemiology of the disease (Freeman et al., 1999; Srinivasanprasad, 1996c). PCR-based techniques have been used to

across DNA polymorphisms in a wide variety of fungi. Random amplification of polymorphic DNA was used to study population genetic structure and host specificity of *Plasmodium cynopteri* (Crombagh et al., 1992) and *Altarium* spp. (Peters et al., 1993). Distinctions between isolates of *C. phaeosporus* from abroad in the U.S. and local ones were yielded with relatively primed PCR (ap-PCR) analysis (Friesman et al., 1996). Molecular techniques such as ap-PCR and RAPD are relatively fast, require only small (μ g) quantities of DNA, do not involve radioactive isotopes, provide randomly assessed loci, and target multiple neutral regions of the genome (Blinchik et al., 1995; Peters and Magnus, 1993). The purpose of this study was to characterize host- and interspecific isolates responsible for their role of crown rot epidemics in Florida, and to evaluate the genetic relationships among isolates of these pathogens to provide insight into the biology of the pathogens and ecology of the disease.

Materials and Methods

Isolates

A collection of 188 strawberry isolates of *Colletotrichum* spp. from anthracnose fruit rot (20 isolates) and *Colletotrichum coccinea* rot (3) of isolates (Table 1) were evaluated. The isolates were collected from 10 strawberry fields within 12 km radius in the Plant City-Davenport area of west central Florida from 1995 to 1999. One of the fields was sampled three times in the same season (Table 1: samples Davenport 9-1, Davenport 9-2, and Davenport 9-3) to obtain sufficient isolates from one field for population genetic analysis. Nine isolates of *C. phaeosporus* (Table 2) from strawberry crown rot lesions and 13 isolates of *C. phaeosporus* from non-strawberry hosts (Table 2) were also included in the

study. These isolates had been previously identified to species level (Table 2).

Mycoscedal cultures were maintained as stored in 20% glycerol at a -80°C freezer.

Mycelium Production and DNA Extraction

Mycoscedal cultures of each isolate were grown on potato-dextrose agar (PDA) at 24°C under continuous fluorescent light for five to seven days. Conidia suspensions were prepared from the cultures in sterile distilled water and 1 ml were inoculated using sterile pipets, to 250 ml flasks containing 100 ml of Buntner Media (4 g yeast extract, 10 g soluble starch, 1 g potassium phosphate, 0.5 g magnesium sulfate, distilled water up to a liter). Cultures were incubated at room temperature under continuous shaking (150 rpm). After three days, mycelium was harvested, mixed with sterile distilled water, filtered through cheesecloth, then freeze dried with a vacuum-dry evaporator (Gene-Son Windstar, USA). The dried mycelium was ground into a powder using sterile glass rods in 10 ml Falcon tubes, and then suspended in 1 ml extraction buffer (70% vol NaCl, 30 mM TrisHCl, pH 8.0, 1% β -mercaptoethanol, and 1% hexadecyltrimethylammonium bromide). Suspensions were incubated at 45°C for 60 min, then 1 ml of chloroform:isoamyl alcohol (24:1 v/v) were added and the mixture emulsified by gently shaking the tubes by hand. After centrifugation at 4°C for 20 min, the supernatant was re-emulsified by hand with 0.5 ml of chloroform:isoamyl alcohol and centrifuged at 4°C for 20 min. The resulting supernatant was mixed with 0.5 volume of isopropanol and centrifuged for 5 min. The DNA precipitate was air dried and re-suspended in 2.0 ml of TE (10 mM Tris, 1mM ethylenediaminetetraacetic acid, pH 7.5). The DNA was re-precipitated with 0.5 volume 7.5 M ammonium acetate ($\text{NH}_4\text{SCl}/\text{GA}_3$) and two volumes

Table 1. Isolates of *Colletotrichum* spp. isolated from damaged strawberry tissue (petiole, calyx or fruit only) at west-coast, Florida

Tissue tested (area)	Source	Year	Number of isolates
Crown	Dover 1 ^a	1988	4
	Dover 8	1990	1
	Dover 8	1990	1
	Dover 9-1 ^b	1990	15
	Dover 9-2 ^b	1990	13
	Dover 9-3 ^b	1990	11
	Dover 21	1987	14
	Dover 24	1989	8
	Dover 24	1987	5
	Dover 24	1989	2
	Dover 26	1991	2
	Dover 17	1987	2
	Dover 18	1990	2
	Dover 25	1990	15
		Sub total	118
Fruit	Dover 2b	1992	3
	Dover 16	1992	2
	Dover 4b	1991	2
	Dover 5	1990	24
	Dover 6b	1990	2
	Dover 7b	1990	2
	Dover 8	1990	3
	Dover 3	1989	1
	Dover 9	1990	6
	Dover 10b	1997	1
		Sub total	50
	Total	168	

^a Isolates of *C. fragariae*.

^b Isolates collected from the same field on three different dates: November 23 (Dover 9-1), December 30 (Dover 9-2), and February 24 (Dover 9-3).

Table 3. Incidence of *Colletes* spp. obtained from non-strobiliferous hosts and used for comparison with *Colletes* spp. recovered from strobiliferous hosts

Locality	Host	Symptom	Incides collected	Species
Arkansas	Woods ¹	Unknown	2	<i>C. glaucoposticollis</i>
Deer, Florida	Sweet gum ²	Unknown	1	<i>C. glaucoposticollis</i>
Honolulu, Florida	Mango	Unknown	3	<i>C. glaucoposticollis</i>
Lake Alfred, Florida	Citrus spp.	Unknown	1	<i>C. aculeatus</i>
Lake Alfred, Florida	Citrus spp.	Unknown	4	<i>C. glaucoposticollis</i>
South Carolina	Peach	Prickles	1	<i>C. aculeatus</i>
South Carolina	Cape	Prickles	1	<i>C. aculeatus</i>
Total			13	

¹ Unknown/first record hosts

² Sweet gum: *Liquidambar styraciflua*

ethanol. After centrifuging (5 min), the pellet was re-suspended in 400 μ L of TE buffer and RNase- (10 mg of RNase per milliliter). After 10 min, DNA was precipitated with 0.1 volume 7.5 M NH₄Cl and 2 volumes ethanol then centrifuged in 14 000 rpm for 10 min. The DNA pellets were re-suspended in 50 to 500 μ L of TE buffer, and stored at -20°C.

ITS Sequencing

All isolates were assigned to species by polymerase chain reaction (PCR) amplification using species-specific primers from the ribosomal DNA internal transcribed spacer (ITS) region (Gonzalezgarcia et al., 1996a, 1996b). PCR amplifications were carried out in 10 μ L reactions containing 2 μ L of DNA, 2 μ L of 10 \times reaction buffer (Life Technologies Inc. IT95 Ready-To-Go), 1.1 μ L of 1.5 mM MgCl₂, 1.0 μ L of dNTP (2 mM), 0.1 U Bsthoenix (0.2 μ L of Ttaq polymerase (5 units/ μ L) and 10.0 μ L of sterile distilled water. Ten μ M (1 μ L) each of the universal internal primer from the ITS4 region (5'-TCTCGGCGTGAATGATATGA-3') and the species-specific primer Orib2 (5'-GGGGAAGCCCTCTGGCGG-3') for *C. ovisense* or the specific primer C₂Tta1 (5'-GAGCCCTCCGCGGCTCCCGGC-3') (10 μ M) for *C. glaucopernix* were used for identification (Gonzalezgarcia et al., 1996a, 1996b). DNA was amplified without initial step of 4 min at 94°C then 34 cycles of 1 min at 94°C, 1 min at 59°C and 2 min at 72°C. DNA was separated by electrophoresis on a 2.5% (w/vol) LE agarose (Pharmacia) in 0.5x TBE buffer (8 mM Tris borate, 0.02 M EDTa, pH 8.0), stained with ethidium bromide and then photographed by transillumination using a Polaroid MP-4 with B1T film (Polaroid Corp., Cambridge, MA).

Enhancement Tests

The pathogenicity of representative strawberry isolates of *Colletotrichum* from across the field isolates and fruit rot (28 isolates) isolates of *C. acrothecium* and *C. gloeosporioides* from our strawberry hosts (13 isolates) and four isolates of *C. gloeosporioides* were bioassayed in laboratory and greenhouse experiments. Isolates for the assays were selected to include representatives from all major biotypes within a dendrogram generated from RAPD and microsatellite markers and included at least two strawberry isolates from each field location (Table 1). Sterile distilled water was used for a control treatment. For each treatment, seeds were harvested from 7-day-old colonies grown on PDA at 18°C under continuous fluorescent light and placed in sterile distilled water to 1×10^6 conidia per ml using a hemacytometer.

The ability of each isolate to produce visible rot symptoms was evaluated by inoculating three greenhouse grown strawberry plants of cultivar ‘Cascadia’ using a 1 ml syringe/plunger equipped with 0.26 mm x 11-mm (26G 1/2) needle. The needle was used to wound and deliver 0.1 ml of freshly made conidial suspensions into the lower third and middle zones. After inoculation, the plants were placed inside a misting chamber at 24 to 26°C for 48 hours and later transferred to a greenhouse at the same temperature for 5 weeks. Plants were evaluated weekly for symptoms characteristic of *Colletotrichum* rot (i.e., wilting and collapse of plant). An isolate was considered to be pathogenic if two of the three inoculated plants wilted and collapsed within 30 days of inoculation. Crown rot from diseased plants were surface sterilized, pasteurized and plated onto a *Colletotrichum* semi-selective medium (14 g of Difco potato dextrose broth, 14 g of Difco agar, 250-mg of nystatin, 100-mg of nystatin-sulfate, 5 mg of sporesin (FL 100 µl of Tergitol), and de-ionized water to 1 liter) (Jurek et al., 2000) for re-isolation and

confirmation of the pathogen. All isolates were evaluated in at least two separate inoculation experiments.

To evaluate fruit pathogenicity, laboratory assays were conducted using fully detached ripe fruit of culture Chassagne. Fungal cultures were grown as previously described. For each isolate, ten detached fruit were inoculated by depositing on the fruit surface 10 μ l of a conidial suspension (1×10^8 conidia/ml) using a 100 μ l pipette. Fruits were placed inside plastic containers (30 x 15 x 10-cm)-on shelves-were racks suspended above 100 ml of water and inoculated for 4 days at 25°C. Each container was wrapped with plastic film (Raychemb Film-11 0.15 mm-thick high density polyethylene). After ten days, the containers were unwrapped and fruit were examined daily for the development of lesions for two additional days.

RAPD and Microsatellite Markers

Thirty-four primers were evaluated for their ability to generate markers amplified polymorphic DNA or microsatellite markers. Four primers were selected for use in this study: (GACA)_n [(GACACAGCAGGACA)GACA] (CACTG)_n [ACTGACTGACTGACTG] (TTC)_n [TCTTGCTGCTGCTGCTG], and DPO-8 [GATGAGCGGC] (Qsonar

Technology). In the case of the primers (GACA)_n, (ACTG)_n, and (TTC)_n, PCR amplifications were done in 20 μ l reactions containing 15.3 μ l of sterile distilled water, 2.7 μ l of 10.3x reaction buffer (100 mM Tris-HCl, pH 8.3, 1.5 mM of (NH₄)₂SO₄, 30 mM MgCl₂, 5% Ficoll 400, 10 mM Dithiothreitol, Life Technology, Inc. 1739 FiedlerDr.), 0.4 μ l of dNTP (2.5 mM P-L Nucleotides), 2 μ l of DNA, 2 μ l (20 μ M) of primer, and 0.3 μ l of Taq polymerase (5 units/ μ l). Samples were overlaid with 30 μ l of mineral oil and DNA was amplified in a programmable thermocycler (MJ-7 PTC-9600, Life Technologies, MA) in an initial step of 5 min at 95°C, then 34 cycles of 1 min at 94°C, 1 min at 40°C

(QACAs), at 40°C (AGTQ)₂ and (TCC)₂ primers, and 1–3 min at 72°C. PCR amplifications using the DPC-8 primer were done in 10- μ l reactions containing 0.2 μ l of sterile distilled water, 0.2 μ l of 10 X reaction buffer (Miles Technology, Inc. 1739 FossilDye), 0.4 μ l of (dNTP) (2.5 mM P-4, bioblocks), 0.1 μ l of DMSO, 0.1 μ l (20 μ M) of primer, and 0.2 μ l of taq-polymerase(3 units). Samples were overlaid with 10 μ l of mineral oil and (DNA was amplified with an initial step of 3 min at 95°C then 34 cycles of 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C. PCR products were separated by electrophoresis as described previously using a 2% (w/vol) agarose (Gibco BRL) in 0.5x TBE buffer. All RAPD and microsatellite primers were tested at least three times for each isolate. Forty different DNA fragments that were consistent PCR products were treated as molecular markers and scored as present or absent.

Data Analysis

The genetic structure of the sampled *Callosobruchus* populations were analyzed and summarized using the straightforward gene group method with arithmetic averages (UPGMA) based on DICE similarity coefficients. Dendrograms were constructed and a coplanar coefficient value was calculated for the phenetic tree generated by UPGMA cluster analysis and the similarity matrix does not need numerical support for the clustering represented in the dendrogram. Analysis was performed using the cluster analysis program NTSYS-PC version 2.0 (Exeter software, Shumate, NY). Further examination of genetic diversity in *C. glabratus* (this review) and *C. maculatus* that not populations were conducted on four subpopulations (Dover 8-1, Dover 8-2, Dover 8-3, and Dover 8) that had a large number of isolates per subpopulation. Research has shown that several populations of *Callosobruchus* spp. can sustain high levels of genetic variability (Ruhle et al., 1999; Christofery et al., 1999; Doniger and Lindey, 1993; Sankaranarayanan et al.,

1996a; Bates, 1992). Therefore, in order to assess relationships among populations it is necessary to measure the proportions of genetic variation within and between populations. Allele frequencies were estimated within each of these four subpopulations (each weighted by population sample size) and the population substructure was evaluated by an analysis of molecular variance (AMOVA; Excoffier et al., 1992). Linkage disequilibrium between pairs of loci was evaluated to test the hypothesis that variation within subpopulations was a result of neutral recombination. In linkage disequilibrium tests, the likelihood of the sample under the hypothesis of no association between loci (random genetic association between alleles) is compared to the likelihood of the sample when recombination is allowed (pairs of loci fail to undergo recombination = linkage disequilibrium). The significance of the observed likelihood ratios is found by computing the null distribution of this ratio under the hypothesis of linkage equilibrium using a permutation procedure. The test is more powerful at detecting departure from equilibrium for higher values of r^2 , the measure of nonrandom (excess) of alleles observed in the sample (Schneider et al., 2000). To increase the detection power of the test, permutations in the program were set so that only polymorphic loci with at least three different combinations of alleles in the sample ($r \geq 3$) were considered. All AMOVA and linkage disequilibrium analyses were performed using the ARLEQUIN program (version 2.0; Guenot and Insaurralde Laboratory, University of Geneva, Geneva, Switzerland).

Results

Sequence-Specific Primers

All ITS isolates (143 from symbiotic and 13 from non symbiotic hosts) of *C. glaucosporoides* and *C. anastomus* were successfully cloned to species by amplification

of a 450- or a 498 bp DNA fragment with one of the species-specific primer sets (Cg/C-Int1 or Ccol2). All but two of the 116 isolates from strawberry crowns produced a characteristic amplification product when the *C. glaucosporus*-specific primer set (Cg/C-Int1 / ITS-4) was used. The remaining two isolates and all 50 fruit rot isolates produced an amplification product when the *C. acutatum* primer set (Ccol2 / ITS-4) was used. Species identities of isolates from non-strawberry hosts were confirmed using the ITS primer (Table 2).

Pathogenicity tests

Scap-like crown isolates (18 of *C. glaucosporus* and four of *C. fragariae*), 33 *C. acutatum* isolates (two from strawberry crowns and 30 from strawberry fruit) and 13 isolates from non-strawberry hosts were evaluated for their ability to produce disease in strawberry. When inoculated into crowns, all 50 isolates of *C. glaucosporus* and the four isolates of *C. fragariae* obtained from crowns produced symptoms characteristic of *Colletotrichum crown rot* (e.g. rotted ends and plant death). The two isolates of *C. acutatum* isolated from diseased crowns did not produce typical crown rot symptoms. Rather, these isolates (and two isolates of *C. acutatum* obtained from strawberry fruit) caused a general slow decline in plant vigor, wilting and dying of older leaves, and stopping of the plant. Eighteen isolates of *C. acutatum* obtained from strawberry fruit and all 13 isolates of *Colletotrichum* spp. obtained from non-strawberry hosts did not produce noticeable symptoms in crowns. Although some plants were severely affected by the disease, all of the plants inoculated with *C. acutatum* isolates in the greenhouse bioassays were still alive after four weeks. No symptoms developed if the plants were inoculated externally. *Colletotrichum glaucosporus* was re-isolated and re-characterized from symptomatic crowns, except when *C. acutatum* was inoculated into the crowns.

All isolates of both *C. glaucopernix* and *C. acuminata* produced typical symptoms of an endemiconic virus on inoculated diamond spruce. First, a n. limited lesions occurred lesions and orange-colored-cracked areas within four days of inoculation. No symptoms developed in the sterile water inoculated controls. Cellulose-ether-glucan particles and *C. acuminata* were re-isolated and characterized from collected fruit. RAPD and Microsatellite Markers

Forty RAPD or microsatellite markers were identified for population genetic comparisons of the isolates. DNA markers ranged in size from ca. 208 to 1366 bp. The number of markers varied for each genus with primer LPC-8, (TCC)₁₄, (ACTG)₁₄, and (GACN)₁₄ producing 12, 51, 18 and 7 scorable markers, respectively.

To facilitate the presentation of the genetic relationships among isolates a dendrogram comprised of 12 representative isolates collected from locusts in Dover 1 (juvenile rat, *C. glaucopernix*), Dover 3-1, Dover 3-2, Dover 3-3 (juvenile rat, *C. glaucopernix*), Dover 5 (adult rat, *C. acuminata*) and isolates from non-locust berry hosts (*C. glaucopernix* and *C. acuminata*) was constructed (Figure 1). This group of isolates clustered all the major clusters observed in the dendrogram produced using the complete dataset (100 strawberry isolates and the 12 isolates sampled from non-strawberry hosts). A bootstrap confidence value of 0.999 was obtained when the phenetic tree generated by UPGMA cluster analysis and the similarity matrix from all tested isolates were compared, supporting the branching points for the major clusters found within the dendrogram. The dendrogram of all 179 isolates (not shown) and the one with 12 representative isolates (Figure 1) produced two major clusters

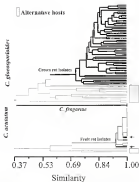


Figure 4. Dendrogram based on RAPD analysis of 32 isolates of *Collessiarchaeum* spp. showing relationships between *Collessiarchaeum* crows red isolates (black box) and *Collessiarchaeum* fruit red isolates (white box) of strawberry and 13 red strawberry isolates (black box with an underline). Two arrows in the cluster of fruit red isolates correspond to the position of two isolates of *C. arvensis* collected from the leaves of plants that did not produce typical symptoms of *Collessiarchaeum* crows red or pathogenicity tests. Dendrogram constructed using the unweighted pair group method with arithmetic mean (UPGMA) based on DSCV similarity coefficients.

The first lineage included all isolates of *C. glaucoperviridis* (as identified by the *CytB* test and the ITS4-primers). This group included two distinct groups: all isolates of *C. glaucoperviridis* from stems (i.e., not all isolates of *C. glaucoperviridis* from non-stem-berry-leaves). The overall similarity coefficient among *C. glaucoperviridis* non-berry leaves not isolates was 0.45, and 0.45 when isolates from non-stem-berry-leaves were excluded. Among all 116 isolates of *C. glaucoperviridis*, only two isolates from subsp. *brun* (Dere 5-3) were distinct for all the molecular markers evaluated (Figure 1). All the other isolates within the *C. glaucoperviridis* lineage had a unique phenotype, showing the high diversity within *C. glaucoperviridis*.

The second lineage (group) for the designates included all *C. acutatum* isolates (identified with the *Coln2* test and the ITS4-primers). The overall similarity coefficient among *C. acutatum* isolates was 0.60. However, the *C. acutatum* group also contained two distinct lineages: all stem-berry-leaves isolates (similarity coefficient of 0.91) and *C. acutatum* isolates from non-stem-berry-leaves (similarity coefficient of 0.30). The two *C. acutatum* isolates obtained from stems were not identified as *C. acutatum* with the ITS primers, grouped with the stem-berry-leaves isolates (Figure 1). Its closest relative in the diversity observed in *C. glaucoperviridis*. 60% of the *C. acutatum* isolates belonged to these clonal groups (similarity coefficient of 0.91) (Figure 1).

The four *C. fergusonii* isolates formed the third major distinct lineage in the dendrogram (Figure 1). The *C. fergusonii* isolates were 34% and 40% similar to the *C. acutatum* and the *C. glaucoperviridis* isolates, respectively. This third lineage was associated with a clonal population composed of historical isolates of *C. fergusonii*, which does not appear to play an important role in most *Colletotrichum* epidemics in

diversity, as indicated by the lack of repeats of the pathogen since the 1980's and the lack of isolates of this species in samples collected for this study.

AMOVA analysis of allele frequencies included one *C. acetosus* fruit rot population (Dover 3) and three *C. glaucopneustes* crown rot populations (Dover 5-1, Dover 5-2, and Dover 5-3). This analysis revealed highly significant differences ($P_{ST} = 0.63$, $P < 0.01$) between the fruit rot population (Dover 3) and each crown rot subpopulation. This means that a large part of the molecular variability (63%) resided within subpopulations. Significant differences were also observed among subpopulations of *C. glaucopneustes* ($F_{ST} = 0.15$, $P < 0.05$). Differences among subpopulations Dover 5-1 and Dover 5-2 ($F_{ST} = 0.34$), Dover 5-1 and Dover 5-3 ($F_{ST} = 0.22$), and Dover 5-2 and Dover 5-3 ($F_{ST} = 0.17$) were all significant ($P < 0.01$). Allele frequencies of these subpopulations of crown rot (for each locus with a mean allele frequency $\geq 5\%$) are presented in Table 3.

Because of the significant differentiations among subpopulations, pairwise linkage disequilibrium was calculated individually for each sub-population of *C. glaucopneustes*. Twenty-four polymorphic loci were used as linkage disequilibrium tests (place first loci at least three different combinations of alleles in the subpopulation) and percentages of linked loci per locus ($P < 0.05$) are presented in Table 4. The numbers of multiple alleles for a particular locus as well as the number of selected loci were greater among *C. glaucopneustes* crown rot subpopulation than the *C. acetosus* fruit rot population (Table 4). Of the 23 positive loci evaluated among crown rot isolates, 17 in subpopulation Dover 5-1, 15 in Dover 5-2, and six in Dover 5-3 were tested. For crown rot isolates, the percentages of linkage between two pairs of multiple-allele loci were

Table 3. Allele frequencies of 74 putative molecular loci generated with RAPD and microsatellite markers from three subpopulations of *Callosobruchus chinensis* on [C: glaucopunctatus] obtained from a commercial seedstore from a west central Florida.

RAPD or microsatellite	Subpopulation			Mean ²
Locus size (bp)	Donor P 1 ¹ (n=15)	Donor P 2 ¹ (n=15)	Donor P 3 ¹ (n=11)	
Primer (GACA)_n				
1.2	0.000	0.000	0.000	0.000
1.8	0.000	0.000	1.000	0.612
6.2	0.000	0.700	0.545	0.707
9.5	1.000	1.000	1.000	1.000
9.7	1.000	1.000	1.000	1.000
Primer (ACTG)_n				
2.1	0.000	0.133	0.000	0.133
1.5	1.000	0.867	1.000	0.945
1.2	0.300	0.867	0.545	0.719
1.8	0.600	0.611	0.555	0.729
8.93	0.600	0.644	0.591	0.652
8.8	0.600	0.611	0.591	0.628
8.6	0.600	0.636	0.625	0.628
8.5	0.600	0.733	0.599	0.628
8.4	0.600	0.700	0.591	0.614
8.3	0.700	0.600	0.500	0.600
Primer GPC-3				
2.8	0.133	0.040	0.000	0.045
2.6	0.000	0.067	0.000	0.023
2.1	0.300	1.000	0.312	0.675
2.7	0.300	0.067	0.636	0.388
1.5	1.000	1.000	0.727	0.905
1.0	0.000	0.000	0.000	0.000
1.2	0.000	0.078	0.000	0.023
8.8	0.529	0.000	0.636	0.388
8.7	0.700	1.000	1.000	1.000
8.6	0.800	0.174	0.000	0.305
8.2	0.000	0.043	0.000	0.017
Primer (TGG)_n				
1.5	0.200	0.533	0.364	0.367
1.1	0.000	0.000	0.182	0.035
1.0	0.600	1.000	1.000	1.000
8.8	0.600	0.600	1.000	0.742
8.2	0.000	0.000	0.000	0.000

¹ Sample sizes are given in parentheses.

² Mean weighted by population or subpopulation sample size.

Table 4 Percentage of linked loci for 11 genome molecular loci generated with RADP and microsatellite markers of three subpopulations of *Colletes strabus* (three red subpopulations: Diver 9-1, Diver 9-2 and Diver 9-3, *C. glaucopunctatus*) and one population of *colletes* from red (Diver 5, *C. arcticum*). A dash means nonsignificant linkage.

RADP or microsatellite	Population or Subpopulation ¹			
	Diver 9-1 (n=22)	Diver 9-2 (n=22)	Diver 9-3 (n=11)	Diver 5 (n=24)
Primer (LACNA)₂				
1-2	-	9.1	-	-
1-8	-	9.1	-	-
2-7	6.3	14.5	18.2	-
2-8	0	0	-	-
Primer (ACTC)₂				
1-5	0	14.5	-	-
1-2	6.3	0	-	-
2-5	12.5	-	-	-
2-8	-	0	27.3	-
3-5	12.5	22.7	-	16.7
3-8	0	9.1	-	-
4-5	0	-	-	1
4-8	18.2	-	-	-
Primer (PCF)₂				
1-4	22.7	-	-	1
1-8	22.7	-	27.3	-
1-7	-	-	27.3	-
1-5	6.3	9.1	-	-
1-2	6.3	14.5	0	1
2-7	-	-	-	20.8
2-5	-	22.7	-	1
2-8	12.5	9.1	0	-
Primer (TCC)₂				
1-5	6.3	9.1	-	-
2-4	18.2	22.7	-	-
2-7	6.3	-	-	1
No. polymorphic loci	17	18	6	3
No. linked loci	4	3	2	0

¹ Sample sizes are given in parentheses

remained between 4% (unlinked locus) to 25% (Table 4). Only two had out of 20 could be analysed for that rat isolate and they were linked as 25% of the samples (Table 4).

Discussion

The results of this study support the hypothesis that anisomycin that rat is caused by *C. caryatum* and *Callosiurechium caryum* rat is caused by *Glossocella caryum* the teleomorph of *C. glaucosporoides*. The large differences in genetic architecture of these species (high diversity and low linkage disequilibrium versus low diversity and high linkage) indicate that the former populations are clonal or nearly so, while the latter species is sexually reproducing.

The ITS primers could not differentiate between *C. glaucosporoides* and *C. faguriae* isolates, due to the uniformity of the replication fragments of genomic DNA generated with the species-specific primers (100 bp). The difficulty encountered while trying to identify isolates of *C. glaucosporoides* and *C. faguriae* with ITS primers was expected due to the lack of significant differences between the two species in the region of the ribosomal DNA, from where the primers were designed (García-Villanova et al., 1996a, 1996b). Our identification of the rat isolate from anisomycin as *C. glaucosporoides* and not as *C. faguriae* is supported by the grouping in our dendrogram analysis. Furthermore, the positive *C. faguriae* isolates in the dendrogram appear to have the usual structure expected for a species that can be differentiated from *C. glaucosporoides* by the absence of a sexual state (Miles, 1982).

All isolates of *C. glaucosporoides* from anisomycin had some of the isolates of *C. caryatum* produced typical crown rot symptoms in greenhouse bioassays. Although *C. caryatum* can potentially cause crown rot like symptoms on anisomycin, the former

decline of the plant-differentiated stresser rot symptoms produced by *C. acroton* from those of *C. gloeosporioides*, when a fast wilting and death of the plant typically occurs. Only *C. gloeosporioides* was recovered from dead marshberry plants, supporting the conclusion that *C. gloeosporioides* and not *C. acroton* is responsible for *Colletotrichum* stresser rot epidemics. Only petiole, recent and older infections are reported in the original description of marshberry diseases caused by *C. fragariae* and *C. gloeosporioides* (Brooks, 1954; Delpe and Lillioelund, 1980; Howard et al., 1992). Systems that are epidemics only developed in the United States after the introduction of *C. acroton* in the 1980's (Howard et al., 1992; Smith, 1998). Most early descriptions of wilting and death rot identify *C. acroton* as the responsible pathogen (Dennis and Bessy, 1948; Eastwold and Gubler, 1980; Howard et al., 1992). Reports of fruit rot epidemics by *C. gloeosporioides* or *C. fragariae* may be unrelated to parasite problems in properly identifying species of *Colletotrichum* based on morphological characteristics (Fennell et al., 2000; Soudanpour et al., 1996). Under extreme conditions *C. acroton* can cause a slow decline and death of marshberry plants (Fennell and Katan, 1987; Fennell et al., 1987) but this is a different disease than the rapid plant wilt and death caused by *C. gloeosporioides* and *C. fragariae* (Brett and Carter, 1966; Leprie 2000). If the stresser wilting and death and *Colletotrichum* stresser rot are to be resolved, a new path must be considered to describe the slow decline associated with stresser rot epidemics by *C. acroton*. If that were the case, the name '*Colletotrichum* slow decline' is suggested for symptoms of marshberry stresser rot associated with *C. acroton*.

Although the results of the stresser/leaves/rot clearly distinguished between isolates of *C. gloeosporioides* (pathogen= wilt and death of the plant) and isolates of *C.*

ascutatus (competing with plants well above water 4 weeks) from strawberry, all the isolates were pathogenic to fruit broomrape. It is likely that ripe detached strawberry fruit are not reliable for determining pathogenicity of *Colletotrichum* spp. on strawberry fruit. Other researchers (Denny-Bishop et al., 1999; Freeman et al., 1996; Smith and Black, 1992) have had similar difficulties in distinguishing pathogens from saprophytes when using detached fruit for broomrape. Therefore, it is reasonable to assume that the results from detached, ripe fruit broomrape using *C. gloeosporioides* crown rot or *C. ascutatus* fruit rot isolates are not indicative of the true pathogenic capability of *C. gloeosporioides* crown rot isolates on fruit, and that these isolates do not cause widespread fruit rot of strawberry under field conditions.

The analysis of DNA markers strongly support the conclusion that *C. gloeosporioides* is the causal agent for *Colletotrichum* stem rot and *C. ascutatus* is the causal agent for anther rot on fruit. With the exception of two *C. ascutatus* isolates, which did not produce typical crown rot symptoms (primary lesions at the dead apices were associated with *C. ascutatus* [fruit rot] and *C. gloeosporioides* [crown rot]). The isolated *C. gloeosporioides* isolates formed a distinctive group in the dendrogram but because few samples were available and no new isolates were obtained from the sampled locations an correlation can be produced from the molecular analysis other than the observation of an apparent closed structure.

Differences detected by the AMOVA analysis among populations of fruit and crown rot were expected given two different species caused the diseases. Genetic diversity among isolates of *C. ascutatus* fruit rot was usually lower than that observed among isolates of *C. gloeosporioides* crown rot. The low diversity in RAPD and

monomorphic markers is attributed to the highly clonal nature of *C. aculeatus*. This lack of diversity meant that fewer loci were detected with multiple copies of alleles using these six isolates (*C. aculeatus*) than those represented in the diverse rat populations (*C. glomerulatus*). This conclusion limited our analysis of linkage disequilibrium to only two out of possible 40 loci in the case of the *C. aculeatus* first rat population. Decreases of lower diversity among microsatellite markers of *C. aculeatus* in comparison with *C. glomerulatus* isolates have been reported in the past using different approaches such as isozyme analysis, arbitrarily primed PCR, A + T Rich DNA, and microsatellite and random characterisation (Boudie et al., 1994; Deshayes and Boudie, 1995; Freeman et al., 1995).

In contrast, the high level of diversity within the *C. glomerulatus* across rat populations is indicative of a sexually reproducing population. Previous research also found high levels of variation among *C. glomerulatus* isolates from microsatellite and other loci (Boudie et al., 1995; Chakrabarty et al., 1997; Freeman et al., 1998; Hudson et al., 1992). Considerable diversity among isolates of *C. glomerulatus* in restriction fragment polymorphisms (RFLP) and mtDNA have been described for isolates exhibiting multiple morphs and other loci (Hudson et al., 1994). Isolates of *C. glomerulatus* pathogenic to *Syllivaster* spp. were highly variable when RAPDs markers were used (Chakrabarty et al., 1997).

It is important to note that sexual reproduction is not the only possible explanation for high diversity in *C. glomerulatus*. Populations greater diversity is also a function of effective population size (N_e), under neutrality theory, large populations maintain higher levels of allelic variation (Kasson, 1982). Diversity is also a function of

molecular evolutionary rate, higher mutation rates can produce a higher standing crop of genetic diversity (Hudson et al., 1992). The age of populations can also influence genetic diversity, as a habitat that is recently colonized will harbor less diversity than an older population, especially if the young population was founded by a few colonizers (Hutchings & Kelly-Kuhl and Clark, 1995). The genetic diversity reported in these findings could also be explained by extremely long periods of independent evolution from a single source that granted the ability to colonize a particular host, or by assuming that pathogenicity on a certain host may have been acquired by a large number of genetically distinct strains (Chakraborty et al., 1997; Hudson et al., 1992). None of these explanations can be excluded with certainty. However, asexual reproduction by somata results in clonal population structures that have distinctive features, such as widespread occurrence of identical genotypes and associations between independent sets of genetic markers (Hollingsworth, 1996). Our results from the coefficients of variability and linkage disequilibrium rates agree with the characteristics of an asexual population for *C. rosea* and *C. fragariae*, but not for *C. glaucoperculae*. The relatively large number of unlinked loci found in the crown rot population demonstrates that at least some of the molecular markers have been under a state of random genetic association or sexual reproduction (linkage equilibrium) (Hill and Clark, 1983). On the other hand, the low levels of recombination (linkage disequilibrium) found among putative loci from the *C. rosea* population, where no unlinked loci were detected, are indicative of an asexual population. Low levels of linkage disequilibrium can also result from the examination of very short regions of the genome that have low levels of recombination or by looking at regions which do not undergo recombination at the same rate as the rest of the genome.

(Hartl and Clark, 1989). Bootstrap markers from RAPD and microsatellite analyses are produced from randomly reassigned loci, and target multiple neutral regions of the genome (Black et al., 1999; Prosser and Malmgren, 1993). The low levels of linkage disequilibrium observed in the *C. aculeatus* population (this loci) are not likely to be the result of ongoing selection of the genome with low levels of recombination. Rather it strongly indicates that clonal processes, such as vegetative production by runners, influenced the first set population evaluated in this study.

The limited diversity seen within the *C. aculeatus* population can also be explained by the use of microclonal transplants from different nursery sources, which increase the diversity of *C. aculeatus* microclonal populations by the addition of different clonal lineages. These clonal lineages can later be dispersed through fields by seeds being carried by beetles, root dispersal or rodents (Blomquist et al., 1999; Legend, 2000; Weiss, 1994) as is suggested by the characteristics of the *C. aculeatus* first set population as well as very low diversity seed as described linked loci (clonal population) as reported here. A similar conclusion has been suggested in studies on the epidemiology of *C. aculeatus* (Gougeon and Baudry, 1998; Baudry and Gougeon, 1999; Fournier et al., 1997; Fournier and Kuhn, 1993; Royen et al., 1992; Wilson et al., 1991). In contrast to the situation described with populations of *C. aculeatus*, first set collections from microclonal may play an important role in the epidemiology of *Colletotrichum* species not associated with *C. glaucoparviflorus* (population highly polymorphic with unrelated loci).

It is interesting that only the sexual stage (*Colletotrichum* spp.) has been observed microclonal (Black et al., 1999; Gougeon and Baudry, 1998; Fournier and

Katon (1977, Miles, 1991) despite our results that demonstrate the strong possibility for the existence of a sexual stage. Postbaccal production in *G. rigaudi* is very sensitive to environmental conditions (Janson, 1992). According to Janson (Janson, 1992), this may have discouraged looking for sufficient strains or to search for the appropriate environmental conditions when looking for the perfect state of *Callosotrichum* spp. The occurrence of sexual individuals for *Callosotrichum* strains not apparently produced by the telomorphs strain (*G. rigaudi*) may be taking place under environmental conditions different than what occurs in their production fields. As it was stated above, it is likely that zoospores are the source of primary infections for *Callosotrichum* diseases of sorghum (Howard et al., 1992; Freeman et al., 1997) and the telomorph stage may be occurring in the nursery.

The resistance that telomorph *Callosotrichum* and *Callosotrichum* strains not are caused by different species *C. arbuscula* and *G. rigaudi* (synonym *C. glomerosporium*) respectively, and the large differences in genetic diversity within each species has important implications for quarantine and management of these diseases. Resistance evaluation was one of the most effective means of controlling disease (Demeyer and Baudry, 1983; Miles, J. L., 1990). In several studies, the expression of disease resistance in *C. arbuscula* has been found to be dependent on the strain of the pathogen (Aguiar et al., 1992; Chakraborty et al., 1993; Demeyer-Baudry, 1997; Demeyer and Baudry, 1998), which may explain why 14 *C. arbuscula* isolates originally recovered from symptomatic strawberry isolates did not cause symptoms in inoculated sorghum but did when tested on fruit. When screening/breeding lines for resistance to *Callosotrichum* that rot, it is probably advisable that breeders use several strains of *C. arbuscula* for the inoculations. For

Colletotrichum crown rot, lesions will tend to use *C. glomerosporium* and likely include a broad selection of isolates as effectively virus symptoms for resistance to crown rot. There are resistant cultivars of strawberry that have shown immunity to anthracnose that are caused by *C. acutatum*, but no completely resistant cultivars are known for *Colletotrichum* crown rot caused by the highly virulent *C. glomerosporium* (Denny et al. 1983; Bessy 1983; Delp and Hultineau, 1986; Freeman et al., 1997; Howard 1992; Miao 1992). Limited research has been conducted to evaluate the importance of sexual reproduction in the life cycle of *C. glomerosporium*, the effect on pathogen virulence, or the potential for genetic exchange between distinct isolates from different geographical regions (Bessy, 1992). Sexual reproduction may make *C. glomerosporium* a more adaptable fungus, and therefore a more difficult pathogen to control. *Colletotrichum* spp. can show different levels of resistance to fungicides (Freeman et al. 1997; Smith and Black, 1992) and additional research should be done regarding recommendations on resistance to teleomorph fungicides.

Although the focus of this study was not to elucidate the role of non-strawberry hosts as epiphytes, it appears that isolates affecting other crops such as *Cornus* spp. or mango do not play a role in epidemics of *Colletotrichum* diseases in strawberry. Hodson et al. (1992) reported that isolates obtained from avocado, banana, mango, and papaya cluster in different groups based on rDNA or mtDNA RFLP patterns suggesting a strong specificity among specific populations of *C. glomerosporium* and their hosts. More observations from different production areas accompanied by better taxonomic tools, must be available in order to verify the applicability of the findings.

CHAPTER 3 CONCLUSION

Colletotrichum species cause anthracnose fruit rot and *Colletotrichum coccineum* resulting in significant yield losses on strawberry worldwide. The literature on *Colletotrichum* diseases on strawberry does not consider *Colletotrichum* spp. (Glomerella spp.) important as pathogens of these diseases. However, the causal agents of anthracnose for epidemics of anthracnose fruit rot and *Colletotrichum coccineum* rot, a serious and important question remains concerning the ecology and population genetics of strawberry diseases caused by *Colletotrichum*. In this discussion, isolates recovered from commercial fields were used to identify the ecology and evaluate the genetic diversity of populations of *Colletotrichum* spp. on strawberry. The potential for *C. glomerosporioides* and *C. coccineum* to overwinter as strawberry plant debris to overwinter under field conditions in Florida was also evaluated to determine its potential as a source of primary inoculum.

The data generated here indicate that anthracnose fruit rot is caused by sexually reproducing populations of *C. coccineum* and *Colletotrichum coccineum* rot is caused by sexually reproducing populations of *Glomerella cingulata* (anamorph *C. glomerosporioides*). These conclusions are important when considering the control of *Colletotrichum coccineum* fruit rot and anthracnose fruit rot. Breeding strawberries resistant to fungal populations of *C. coccineum* (anthracnose fruit rot) may be more than developing resistance to *C. glomerosporioides* (*Colletotrichum coccineum*) where significant levels of genetic diversity was detected. A sexually reproducing pathogen population

such as *C. gloeosporioides* has greater potential to overcome resistance genes through recombination of advantageous genes than an annually produced population. The presence of strawberry genotypes highly resistant to anthracnose does not null the lack of resistant genotypes to *Colletotrichum coccineum* nor supports these observations. Given that the pathogen responsible for either fruit or crown diseases can now be identified based on observed symptoms, control measures specific for each pathogen can be developed and implemented. For example, *C. acutatum* is consistently resistant to benzimidazoles whereas *C. gloeosporioides* isolates are variable in sensitivity. Because fruit rot is only caused by *C. acutatum*, benzimidazole fungicides cannot control this disease. However, benzimidazole-type fungicides may control *Colletotrichum coccineum*.

It is important to clarify to the grower that *C. acutatum* can also cause a slow decline and death of strawberry plants that can be confused with *Colletotrichum coccineum*. However, the symptoms of this disease, such as stunting and slow decline, are different from the rapid plant wilt and death caused by *C. gloeosporioides*. Perhaps this disease should involve a different name such as "*Colletotrichum slow decline*".

Populations of *C. gloeosporioides* or *C. acutatum* do not overwinter on bare strawberry plants in Florida. Populations of *C. acutatum* and *C. gloeosporioides* declined to undetectable levels within 30 to 40 days of burial of crown tissue. This time period is long enough to eliminate this potential overwinter source because it is shorter than the 120 to 180 days time period that normally elapses between the end of a strawberry season and the setting of new plants for next season. However, due to the potential for survival of crown lesions for over 30 days as well as preserved strawberry debris, it is prudent that growers incorporate plant material at the end of the season to

allow sufficient time to allow decomposition of the plant tissue and elimination of inoculum.

Because *Colletotrichum* species do not overwinter in soil or plant debris in Florida, other sources of inoculum such as non-strawberry hosts or infected transplants are the potential sources of inoculum for *Colletotrichum* diseases. The objective of this study was not to describe the role of non-strawberry hosts as sources of *Colletotrichum* diseases in strawberry. However, the separation in the dendrogram of *Colletotrichum* isolates recovered from symptomatic strawberries from isolates recovered on non-strawberry hosts suggested that non-strawberry hosts do not play a role as sources for epidemics of *Colletotrichum* spp. in strawberry. This conclusion corresponds with hypotheses of host specificity described for other crops such as mangoes, avocados, and citrus which are also affected by *Colletotrichum* diseases. Transplants are the most likely sources of inoculum for epidemics of anthracnose (but not rot) and *Colletotrichum* crown rot in strawberry. The fact that strawberry plants are mainly propagated from the distribution of infected roots at their nurseries in forcing fields.

Through the use of molecular and molecular tools, a better understanding of the biology of *Colletotrichum* species pathogenic to strawberry has been obtained. Some selective media, bioassays, and isolation and recovery of fungal isolates infecting plant tissue are some of the traditional methods used to study the survival ability of *Colletotrichum* spp. in strawberry plant debris. Similarly, molecular approaches involving RAPD / up-PCR markers, species-specific primers, and PCR have helped to describe the strategy of anthracnose (but not rot) and *Colletotrichum* crown rot and to integrate the population genetics and biology of the pathogens causing these diseases.

Additional research is needed to better characterize the epidemiology of *Colletotrichum* diseases on strawberry. Testing recombination among *C. gloeosporioides* isolates from strawberry and other hosts, evidence for transplants moving as the primary source of inoculum for seedlessness fruit rot and *Colletotrichum* crown rot as introduced fungi and further evaluation of the role of *Colletotrichum* isolates from non-strawberry hosts are some important areas for potential research.

APPENDIX RAPD AND MICROSATELLITE MARKERS

Table 5. Presence (+/absence[-]) of putative molecular loci ordered by molecular weight (bp) generated by PCR with (GACA)₄ and (ACTC)₄ primers from seven oil *n*-data (1) *C. glomerata* as identified by ISS primers.

Locus#	(GACA) ₄							(ACTC) ₄									
	100 bp	150 bp	200 bp	250 bp	300 bp	350 bp	400 bp	100 bp	150 bp	200 bp	250 bp	300 bp	350 bp	400 bp	450 bp	500 bp	550 bp
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PG-41A	+	+	+	+			+	+	+		+		+	+	+	+	+
PG-41B	+	+	+	+	+	+		+	+	+	+	+	+	+	+		+
PG-42A	+	+	+	+			+	+	+	+		+	+	+		+	
PG-42B	+	+	+	+			+	+	+	+		+	+	+	+	+	+
PG-43A	+	+		+	+	+	+	+	+	+		+		+	+		
PG-43B	+	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
PG-44C	+	+	+	+			+	+	+	+	+		+		+		
PG-45B	+			+			+	+	+	+	+		+		+		
PG-45C	+	+		+	+	+	+	+	+	+		+		+			
PG-45D	+	+		+	+	+	+	+	+	+		+		+			
PG-45E	+	+		+	+	+	+	+	+	+		+		+			
PG-45F	+	+	+	+	+		+	+	+	+		+	+	+			
PG-45G	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45H	+	+	+	+	+		+	+	+	+	+		+		+	+	+
PG-45I	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45J	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45K	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45L	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45M	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45N	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45O	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45P	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45Q	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45R	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45S	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45T	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45U	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45V	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45W	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45X	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45Y	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-45Z	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46A	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46B	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46C	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46D	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46E	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46F	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46G	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46H	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46I	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46J	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46K	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46L	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46M	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46N	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46O	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46P	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46Q	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46R	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46S	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46T	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46U	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46V	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46W	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46X	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46Y	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-46Z	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47A	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47B	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47C	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47D	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47E	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47F	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47G	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47H	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47I	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47J	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47K	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47L	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47M	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47N	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47O	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47P	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47Q	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47R	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47S	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47T	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47U	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47V	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47W	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47X	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47Y	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-47Z	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48A	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48B	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48C	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48D	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48E	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48F	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48G	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48H	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48I	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48J	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48K	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48L	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48M	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48N	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48O	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48P	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48Q	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48R	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48S	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48T	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48U	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48V	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48W	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48X	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48Y	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-48Z	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-49A	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-49B	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-49C	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-49D	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-49E	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-49F	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-49G	+	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+
PG-49H	+	+	+	+	+	+											

Table 1. Presence (+) / absence (-) of putative molecular ions ordered by molecular size (kg) generated by PCR, with CPC_{10} and FDC_{10} , proteins from cross-react isolates of *C. glabrata* as identified by ITS primers.

Isolate	CPC ₁₀												Protein FDC ₁₀											
	15	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	
	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	kg	
P00-01A			+		+	+		+	+	+	+			+	+	+	+			+			+	
P00-01B					+	+		+	+	+	+				+	+	+	+		+	+	+	+	
P00-01A			+	+	+	+	+	+	+	+	+				+	+	+	+		+			+	
P00-01B			+	+	+	+	+	+	+	+	+				+	+	+	+		+			+	
P00-01A					+	+			+	+	+				+	+	+	+					+	
P00-01B					+	+			+	+	+				+	+	+	+					+	
P00-01C					+	+			+	+	+	+			+	+	+	+					+	
P00-01D			+	+	+	+	+	+	+	+	+				+	+	+	+					+	
P00-01E			+	+	+	+	+	+	+	+	+				+	+	+	+					+	
P00-01F			+	+	+	+	+	+	+	+	+				+	+	+	+					+	
P00-01G					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01H					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01I					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01J					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01K					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01L					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01M					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01N					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01O					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01P					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01Q					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01R					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01S					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01T					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01U					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01V					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01W					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01X					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01Y					+	+	+	+	+	+	+				+	+	+	+					+	
P00-01Z					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02A					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02B					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02C					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02D					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02E					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02F					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02G					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02H					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02I					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02J					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02K					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02L					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02M					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02N					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02O					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02P					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02Q					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02R					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02S					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02T					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02U					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02V					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02W					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02X					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02Y					+	+	+	+	+	+	+				+	+	+	+					+	
P00-02Z					+	+	+	+	+	+	+				+	+	+	+					+	

Table 11. Percentages (%) of positive molecular tests colored by molecular test type generated by PCR with OPG-9 and (TCC)₂ primers. True detection isolation of *C. parvum* is identified by ITS primers.

Isolate	OPG-9												(TCC) ₂											
	10	12	14	16	18	20	22	24	26	28	30	32	10	12	14	16	18	20	22	24	26	28	30	32
	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
P04-041	+												+	+	+	+	+							+
P04-041													+	+	+	+	+							+
P04-042	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+
P04-045	+	+											+											+
P04-054													+	+	+	+	+							+
P04-055	+												+	+	+	+	+							+
P04-058	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+
P04-057													+	+	+	+	+							+
P04-058	+	+	+	+	+								+	+	+	+	+							+
P04-059	+	+	+	+	+								+	+	+	+	+							+
P04-070													+	+	+	+	+							+
P04-071													+	+	+	+	+							+
P04-072	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+
P04-073	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+
P04-074	+												+	+	+	+	+							+
P04-075	+												+	+	+	+	+							+
P07-023	+	+	+	+	+								+	+	+	+	+							+
P07-034	+	+	+										+	+	+	+	+							+
P07-036	+	+											+	+	+	+	+							+
P07-037													+	+	+	+	+							+
P07-038	+	+	+										+	+	+	+	+							+
P07-039	+	+	+										+	+	+	+	+							+
P07-044													+	+	+	+	+							+
P07-140													+	+	+	+	+							+
P07-141	+	+	+										+	+	+	+	+							+
P07-142	+	+	+	+	+								+	+	+	+	+							+
P07-143	+	+	+	+	+								+	+	+	+	+							+
P08-144	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+
P08-145	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+
P08-146	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+
P08-150	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+
LS09-19	+	+	+	+	+								+	+	+	+	+							+
LS09-18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+
P08-02	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+
P08-0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+							+

APPENDICES

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BIOGRAPHICAL SKETCH

Alonso R. Uribe Padilla was born in San José, Costa Rica, on August 11, 1964. He graduated with a Bachelor of Agriculture (major in crop production) from the University of Costa Rica, San José, in 1982. In 1984, he completed a post-baccalaureate in Engineer in Agricultural Sciences at the same university. From 1983 to 1986, he worked as a permanent member of the Postharvest Laboratory of the Agriculture Research Center of the University of Costa Rica. From 1986 to 1990 he was enrolled in a MSc program (crop protection) at the University of Costa Rica. In 1989, he was awarded a scholarship by the International Development Agency of the United States of America to pursue graduate studies within United States. In 1993, after obtaining a Master of Science (equivalent with a master in plant pathology) from the Horticulture Department of the University of Florida he returned to Costa Rica to work as an advisor for non-governmental organizations (NGO's) and as a packing house manager of an important mango firm in the north of Costa Rica. In 1994, he got the permission from the G. E. Legard to obtain his Ph.D. and transfer his knowledge with the principles and uses of the science of plant diseases working and researching the epidemiology and general biology of *Colletotrichum* spp. associated with strawberries. After obtaining his degree, he plans to continue his goal of looking for an integrated understanding of agricultural production systems that includes pre- and post-harvest handling of products as well as the ecological and sociological aspects of food production.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy


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